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# CLINICAL AND EXPERIMENTAL

## SALMONELLA SUIPESTIFER INFECTION

### REPORT OF A CASE

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THE hog cholera bacillus was described by Salmon and Smith in 1885. It was later named *Salmonella suipestifer*. In 1902 Longcope published a report of two cases of human infection of a typhoidlike nature caused by a "paracolon" bacillus. Further studies with the organism proved it to be a member of the hog cholera bacillus group. This was the first report of human infection of *Salmonella suipestifer*. Since that time several outbreaks of food poisoning by the organism have been recognized, and meanwhile a number of sporadic cases have been recorded. Harvey has reviewed the literature and reported 21 cases of human infection. He states that three outbreaks of food poisoning and a total of 18 sporadic cases have been reported in this country.

In an outbreak of the infection due to a known source, the clinical picture is usually similar in all the cases. After an incubation period of from six to twenty-four hours, the patient is taken rather acutely ill with violent gastrointestinal symptoms, headache, and fever. In severe cases, chills, prostration, and stupor may occur. The acute symptoms usually subside within a few days, although convalescence may not be complete before ten to fourteen days. Recovery has been the rule and the mortality has been low. In such outbreaks the organisms have been isolated from a variety of foods.

The sporadic cases, which have been reported, present a somewhat different clinical picture. The disease occurs with equal frequency in both sexes, and is more frequent in the first three decades of life. The reported mortality of

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from 20 to 60 per cent in sporadic cases of hog cholera infection is considerably higher than in epidemic outbreaks of Salmonella food poisoning.

Two fairly well-defined types of sporadic *Salmonella suipestifer* infection are recognized: (1) an uncomplicated bacteriemia or typhoidlike infection, and (2) the cases with pulmonary involvement. In the former, except for sudden stormy onset, the clinical picture is not unlike that of typhoid fever. Chills are not uncommon and fever is usually marked, reaching 104° to 105° F. and lasting for several days, depending upon the outcome. Anorexia is common and nausea and vomiting may occur. Diarrhea occurs a little more often than constipation. The patient often complains of generalized aching and abdominal pain and distention are not infrequent.

Physical findings are not characteristic, but the picture of an acute blood-stream infection is often present. The diagnosis rests upon agglutination test and blood cultures, and without these the disease is probably diagnosed as typhoid or paratyphoid infection. Involvement of the bones and joints with a pyarthrosis and osteomyelitis is not unusual.

In the cases characterized by a pulmonary involvement, pneumonia, either of the lobar or lobular type, pleurisy with effusion, or empyema may occur. Approximately one-third of the reported sporadic cases have had pulmonary involvement and approximately 40 per cent of these have died. The symptoms and findings in these cases usually point to the pulmonary condition and unless search is made for the organism, the nature of the infection may go unrecognized. Many of these will have an accompanying blood-stream infection and the organism can usually be found in the sputum.

Incidents of infection by *Salmonella suipestifer* have been reported complicating various surgical procedures, and involvement of other tissues in the body, such as the meninges, the endocardium, and the urinary tract has been reported.

The blood picture is not characteristic. In the typhoidlike cases there is a tendency to a leucopenia. With evidence of localizing infection there usually is a leucocytosis. The organism is a member of the same family as the paratyphoid bacilli and cross agglutination with these organisms may give confusing results. The organism remains in the blood stream for a relatively long period and is easily grown on culture media. There are no specific pathologic lesions. Enlargement of the spleen and liver may occur. Petechial hemorrhage and cloudy swelling of some of the viscera are common. There usually are no lesions in the gastrointestinal tract in the sporadic cases.

### CASE HISTORY

Patient was a white female, aged 44 years, and the mother of five children. She was admitted to Johnston-Willis Hospital, April 21, 1937, on the service of Dr. I. H. Goldman for whom she was seen. She was taken rather suddenly ill with chills and fever ten days prior to admission. On the following day she developed cough and pain in the chest. The cough very promptly became productive of yellowish sputum. The pain seemed to arise under the sternum and radiate downward and along the margin of the lower ribs. It was accentuated by deep breathing and coughing. There had been no further chills since the day of the onset, but fever had persisted. Appetite had been poor, but there had been no nausea, vomiting, diarrhea, or constipation. About five days prior to admission, the patient began to notice some frequency of urination and nocturia. About two weeks

before onset of present illness the patient had a "cold" from which she recovered uneventfully. Several months previously, because of a protracted cold, her chest had been x-rayed and was negative. Patient stated that she had always been in good health, but that she had always been small, her average weight being about 105 pounds. The remainder of the history was not significant.

Physical examination revealed a poorly nourished white female, who was quite alert and somewhat apprehensive. She coughed frequently and expectorated thick yellowish sputum. Upon coughing she complained of pain in the middle of her chest in front, running down along the lower ribs on the left. Respirations were slightly accelerated and possibly a little shallow, but there was no marked dyspnea. The skin was warm and moist, the temperature being 103°. There was no evidence of edema, cyanosis, jaundice, or any other abnormality of the skin. Patient had the appearance of one acutely ill. The head was normal as were the eyes and ears. The mucosa of the nose was somewhat red. The tongue was heavily coated, and the gums and teeth were partially covered with sordes. The pharynx was red; the blood pressure 100/70, and the pulse 128 and regular. Heart was normal. Lungs: left, normal. Over the right base posteriorly, low in the axilla, there was a small area of distant bronchovesicular breathing, with a few moist and crepitant râles. The remainder of the examination was essentially negative.

*Laboratory.*—Several repeated urinalyses revealed one significant finding, namely, the presence of innumerable pus cells. The blood work showed a moderate anemia of the microcytic variety, the hemoglobin estimations averaging 65 per cent and red blood count 3,500,000. On admission April 21, 1937, the total white blood count was 8,200, with 70 per cent polys. The highest white blood count was noted April 25, 1937, and was 14,400, with 91 per cent polys. On May 4, 1937, the white blood count was 5,500, with 79 per cent polys. Blood Wassermann was negative, as was routine blood chemistry.

Patient's chest was x-rayed revealing some increase in hilum shadows and in the lower bronchial trunks as well as some infiltration and a small pleural effusion at the right base.

Althought there was evidence of a pulmonary lesion, it was not that of a true pneumonia, and we did not feel that the pulmonary pathology, nor that in the urinary tract, would account in whole for the patient's symptoms.

Routine agglutinations for all of the obscure fevers were negative. Blood cultures and malaria smears were also negative.

The patient continued to run a rather acute course with a septic type of fever, reaching 104° to 105° F. each day. About a week after admission her cough and chest pain became much less severe and almost completely disappeared. In spite of the alleviation of these symptoms the patient's general condition became progressively worse.

On May 1, 1937, she developed large hemorrhagic areas on the buttocks and elbows. These rapidly spread and became necrotic in their centers.

On May 1, 1937, a blood culture taken two days previously revealed the presence of a motile bacillus, which was identified as *Salmonella suipestifer*. Previous blood cultures had been negative. Blood cultures were then taken on three successive days, all of which revealed the presence of the same organism.

The patient's condition became progressively worse and she died May 4, 1937.

## SUMMARY

A case of *Salmonella suipestifer* bacteriemia is reported. The patient had a pulmonary infiltration as well as a small pleural effusion, both of which disappeared before death. Blood cultures taken on the seventeenth day of the illness were positive. Three subsequent blood cultures were also positive. The condition terminated fatally on the twenty-third day.

## REFERENCES

Longcope, W. T.: Paracolon Infection, Together With the Report of a Fatal Case, With Autopsy, Am. J. M. Sc. **124**: 209, 1902.

Harvey, A. N.: Salmonella Suipestifer Infection in Human Beings, Arch. Int. Med. **59**: 118, 1937.

# ASCORBIC ACID IN THE BLOOD AND URINE AFTER THE INTRAVENOUS INJECTION OF SODIUM ASCORBATE*

## A Clinical Test for Determining Vitamin C Deficiency

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THE isolation of ascorbic acid, accompanied shortly afterwards by its synthesis and large scale production, has made possible the use of this substance in clinical experiments. Such experiments became warranted when it was shown (Harris, Ray, and Ward[1]) that ascorbic acid ingested in great quantities was excreted by the kidneys quite rapidly, and that its administration in amounts as high as 200 mg. per kg. weight was followed by no ill effects (Rohmer, Bezssonoff, Stoerr, and Perier[2]). Thus unlike the sterol containing vitamins, the water-soluble vitamins [ascorbic acid (vitamin C), thyamin (vitamin $B_1$), riboflavin (vitamin $B_2$)] can be given to patients safely in quantities many times as great as the curative doses; all three are readily excreted by the kidneys, and no accumulation of these vitamins is observed. This ready excretion, on the other hand, will produce conditions of vitamin deficiency after the vitamin intake is restricted.

The urinary excretion of ascorbic acid is mainly a function of the body storage, or degree of saturation, which in turn is dependent upon the previous dietary history and demands of the organism (Johnson and Zilva[3]). Therefore, the rate of excretion of added ascorbic acid was determined in an attempt to detect clinical cases of vitamin deficiency, the widespread presence of which is now recognized specially as a complication of those diseases (gastrointestinal diseases, nephritis, diabetes) where diets poor in vitamin content have been prescribed. While these studies were in progress, there have been published reports on the excretion of ascorbic acid after the administration of test doses, which will be discussed later.

In the studies to be reported here, sodium ascorbate was injected intravenously into patients in a dosage of 10 mg. per kg. weight, the ascorbic acid content in the blood and urine being determined at different intervals.

### METHODS

The sodium ascorbate was weighed the day previous to the injection, sealed in a test tube, and kept in a 110° C. oven for ten minutes. In the morning, after withdrawing the control sample of blood, the sodium ascorbate (dissolved

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in a small volume of distilled water, 0.3 c.c. per 100 mg. of sodium ascorbate) was injected intravenously. Three samples of blood were taken five minutes, one hour, and two hours later. For the urinary excretion, a twenty-four-hour urine control was previously collected. After the injection, three separate specimens were collected—three hours, eight hours, and twenty-four hours after the injection—and their ascorbic acid content determined.

*Determination of Ascorbic Acid.*—Reagents. (1) *Stock dye:* 25 mg. of 2,6-dichlorophenol indophenol (La Motte Chemical Products Co., Baltimore) were dissolved in 50 c.c. warm distilled water and filtered. It was standardized with acidified ascorbic acid (1 c.c. of a solution containing 5 mg. in 10 c.c. water plus three drops of the trichloracetic-metaphosphoric acid mixture) just before use, and was never kept more than two days. The ascorbic acid has to be freshly prepared because of its rapid oxidation by traces of copper (Barron, DeMeio, and Klemperer[4]). (2) *Dilute dye:* 2 c.c. of the stock dye were diluted to 25 c.c. with distilled water. (3) *Trichloracetic-metaphosphoric acid mixture:* 1.2 gm. of metaphosphoric acid ground in a mortar immediately before use were dissolved in 40 c.c. of a solution of 12.5 gm. per cent of trichloracetic acid. (4) *Approximately 5 N $H_2SO_4$.* (5) 8-hydroxyquinoline.

*In the blood samples,* the ascorbic acid content was estimated as follows: oxalated blood was centrifuged in a cold room (3° C.); 2 c.c. of the plasma were added to a centrifuge tube containing 6 c.c. of the trichloracetic-metaphosphoric acid mixture, shaken together, and centrifuged. Three cubic centimeters of the supernatant fluid (equivalent to 1 c.c. of plasma) were titrated with the *dilute dye* until the appearance of a pink color, stable for half a minute.

*In the urine specimens,* the ascorbic acid was titrated with the stock dye after the addition of four drops of the trichloracetic-metaphosphoric acid mixture. For the twenty-four-hour sample as well as the control twenty-four-hour period sample, the urine was collected in a bottle containing 25 c.c. of 5 N $H_2SO_4$ plus about 50 mg. of 8-hydroxyquinoline and kept in the icebox until the moment of the titration. The "three hour" and "eight hour" samples were titrated after acidification as soon as received. (The oxidation of ascorbic acid in the urine, a very slow process, is due to the copper contained in the urine and to bacterial decomposition. This oxidation may be largely retarded by acidification; addition of 8-hydroxyquinoline prevents the oxidation by copper (Barron, Barron, and Klemperer[5]). If toluol is added, the urine may be kept for twenty-four hours at room temperature with no loss of ascorbic acid, as shown by numerous tests in our laboratory.

*The Rate of Disappearance of Ascorbic Acid From the Blood.*—The 81 patients on whom the rate of removal of ascorbic acid from the blood was determined were divided into three groups: I, those who excreted in two hours 90 per cent or more of the ascorbic acid injected; II, those who excreted from 80 to 90 per cent; III, those who excreted less than 80 per cent (Tables I, II, III). In Fig. 1 are given representative examples of these three groups. As found by Taylor, Chase, and Faulkner,[6] and Wright, Lilienfeld, and Mac Lenathen,[7] the ascorbic acid disappeared from the blood quite rapidly for the

TABLE I

RATE OF DISAPPEARANCE OF ASCORBIC ACID FROM THE BLOOD

GROUP I

Ninety Per Cent Excretion or Over, Two Hours After the Injection of 10 mg. Sodium Ascorbate per kg. Weight

| DIAGNOSIS | DATE | ASCORBIC ACID IN BLOOD BEFORE TEST MG. PER 100 C.C. | PER CENT EXCRETION |
|---|---|---|---|
| Urticaria | Jan. 15, 1937 | 2.76 | 100 |
| Addison's disease | Jan. 15, 1937 | 3.05 | 100* |
| Asthma, morphine poisoning | March 4, 1937 | 2.09 | 100 |
| Cirrhosis of the liver | April 9, 1937 | 0.375 | 100 |
| Carcinoma—stomach | April 7, 1937 | 0.77 | 100 |
| Adrenal tumor | April 1, 1937 | 1.32 | 100 |
| Neurosis | March 5, 1937 | 0.332 | 100 |
| Peripheral neuritis | Dec. 17, 1936 | 0.30 | 99.1 |
| Peptic ulcer | March 3, 1937 | 0.698 | 98.0 |
| Ulcerative colitis | Feb. 4, 1937 | 0.377 | 98.0 |
| Intestinal neurosis | March 18, 1937 | 0.744 | 97.4 |
| Right third nerve paralysis—gallstones | March 4, 1937 | 0.475 | 97.6 |
| Anxiety state | Feb. 10, 1937 | 0.297 | 97.0 |
| Pulmonary tuberculosis | Feb. 5, 1937 | 0.332 | 97.1 |
| Addison's disease | Jan. 29, 1937 | 2.074 | 97.1* |
| Nonspecific ulcerative colitis | Jan. 12, 1937 | 0.412 | 97.1 |
| Diabetes mellitus | Nov. 24, 1936 | 1.06 | 95.1 |
| Peritonitis (tuberculosis) | Feb. 4, 1937 | 0.377 | 95.6 |
| Streptococcus sore throat | April 10, 1937 | 0.622 | 95.8 |
| Left sciatica and arthritis of spine | April 7, 1937 | 0.496 | 95.9 |
| Carcinoma of the lung | March 10, 1937 | 0.325 | 95.6 |
| Duodenal ulcer | Feb. 15, 1937 | 0.482 | 94.7 |
| Chronic glomerular nephritis | March 23, 1937 | 0.723 | 93.2 |
| Subclinical scurvy | June 4, 1937 | 0.552 | 96.4 |
| Alcoholic polyneuritis | Sept. 24, 1937 | 0.265 | 96.5 |
| Alcoholic polyneuritis | June 18, 1937 | 0.711 | 95.6 |
| Cirrhosis of the liver | July 12, 1937 | 0.417 | 91.8 |
| Carcinoma—stomach | Dec. 1, 1936 | 0.672 | 93.1 |
| Duodenal ulcer, stenosis | Nov. 25, 1936 | 0.394 | 93.1 |
| Diabetes mellitus | March 3, 1937 | 0.496 | 92.7 |
| Hyperthyroidism | Dec. 22, 1936 | 0.970 | 92.4 |
| Rheumatoid arthritis | March 2, 1937 | 0.332 | 90.9 |
| Chronic cholecystitis | Feb. 10, 1937 | 0.48 | 90.2 |
| Pernicious anemia | Jan. 26, 1937 | 0.765 | 91.2 |
| Kidney stone—streptococcus throat | March 30, 1937 | 0.810 | 90.1 |
| Diabetes mellitus | June 6, 1937 | 2.31 | 90.9 |
| Average ascorbic acid content in the blood (The ascorbic acid saturated patients not included) | | 0.708 | |

*Saturated previous to test.

first two hours, the rate of excretion afterwards becoming extremely slow. As all the tissues and body fluids contain ascorbic acid, we may safely assume that when ascorbic acid is injected into the blood it freely diffuses throughout the capillary bed, being thus partly excreted by the excretory glands (kidneys, sweat glands, salivary glands) and partly stored by the other tissues. From a study of these three groups of patients we have to conclude that the rate of disappearance from the blood of the injected ascorbic acid has no relation either with the ascorbic acid content of the blood previous to the injection (average ascorbic acid content in the blood of group I, 0.708 mg.; of group II, 0.787 mg.; of group III, 0.664 mg. per 100 c.c.) or with the degree of saturation. One patient with as low an ascorbic acid content in the blood as 0.265 mg. per 100 c.c. re-

moved from the blood 96 per cent of the injected vitamin in two hours, while another with an ascorbic acid content in the blood of 0.126 mg. per 100 c.c. excreted in two hours only 63.2 per cent. Even after the patients were saturated with ascorbic acid by the administration of large doses of it previous to the test, we found two of these patients (Addison's disease) eliminating 100 and 97 per cent of the injected ascorbic acid, while another patient (Addison's disease) excreted only 73.8 per cent. Obvious cases of vitamin deficiency were found in both the fast and the slow excreting groups. A study of the rate of disappearance of ascorbic acid from the blood has, we believe, no significance for determining the ascorbic acid requirements of the human body or detecting vitamin deficiencies.

*The Urinary Excretion of Ascorbic Acid.*—Harris, Ray, and Ward[1] were the first to demonstrate a correlation between the urinary excretion of ascorbic

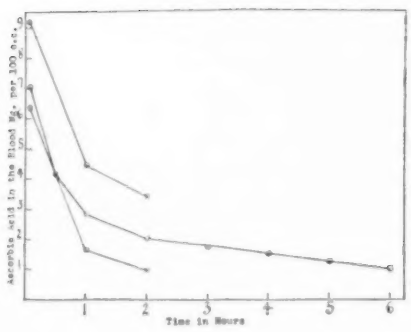


Fig. 1.

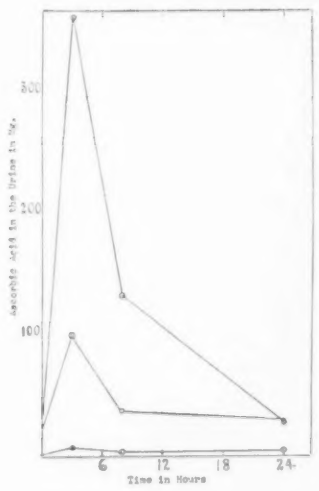


Fig. 2.

Fig. 1.—The three types of ascorbic acid disappearance from the blood after the injection of 10 mg. per kg. weight.

Fig. 2.—The three types of urinary excretion of ascorbic acid injected intravenously.

acid and the dietary intake. Their observations have been confirmed by a number of investigators who have studied the ascorbic acid output either after ingestion of ascorbic acid (Johnson and Zilva,[3] Van Eekelen[8]), or after its injection (Hawley and Stephens,[9] Sendroy and Schultz,[10] Finkle,[11] and Wright, Lilienfeld and Mac Lenathen[7]). It is thus generally accepted that the urinary output or response to a given dose is a function of the body storage or degree of saturation which in turn is dependent upon the previous dietary history and the demands of the organism.

The 64 miscellaneous patients whose urinary output was followed have been listed in three groups: I, those excreting 30 per cent or more in twenty-four hours; II, those excreting from 10 to 30 per cent; III, those excreting 10 per cent or less (Tables IV, V, and VI). In Fig. 2 are given representative examples of these three groups. In general, those belonging to the first group

TABLE II

RATE OF DISAPPEARANCE OF ASCORBIC ACID FROM THE BLOOD

GROUP II

From 80 to 90 Per Cent Excretion

| DATE | DIAGNOSIS | ASCORBIC ACID IN BLOOD BEFORE TEST MG. PER 100 C.C. | PER CENT EXCRETION |
|---|---|---|---|
| Feb. 8, 1937 | Adenoma of bronchus? Syphilis | 0.569 | 89.6 |
| April 1, 1937 | Osteo-arthritis—fingers | 0.498 | 89.0 |
| Jan. 11, 1937 | Addison's disease | 0.412 | 89.4* |
| Jan. 15, 1937 | Addison's disease | 0.356 | 87.3* |
| Nov. 20, 1936 | Functional colitis | 0.386 | 88.7 |
| Jan. 26, 1937 | Diabetic gangrene | 0.755 | 88.6 |
| Jan. 18, 1937 | Peptic ulcer | 0.38 | 88.6 |
| March 12, 1937 | Cathartic colitis | 1.05 | 88.5 |
| Dec. 15, 1936 | Polycythemia | 1.128 | 87.6† |
| Feb. 19, 1937 | Pernicious anemia | 1.544 | 87.8 |
| April 6, 1937 | Arteriosclerotic heart disease | 0.536 | 87.2 |
| Jan. 20, 1937 | Mixed psychoneurosis | 0.695 | 86.7 |
| Feb. 11, 1937 | Anxiety neurosis | 1.61 | 85.9 |
| Jan. 25, 1937 | Diabetic arteriosclerosis | 0.45 | 85.2 |
| Feb. 11, 1937 | Irritability of colon—allergic rhinitis | 0.924 | 85.7 |
| Nov. 19, 1936 | Hypothyroidism | 0.682 | 84.7 |
| Nov. 17, 1936 | Amyothrophic lateral sclerosis | 0.94 | 84.1 |
| April 28, 1937 | Polycythemia | 0.385 | 86.0 |
| Dec. 16, 1936 | Bowen's disease | 1.90 | 84.0 |
| Feb. 26, 1937 | Cardiac decompensation | 0.594 | 83.4 |
| April 6, 1937 | Rheumatic heart disease | 0.745 | 82.0 |
| Dec. 3, 1936 | Cholelithiasis | 0.78 | 82.1 |
| Jan. 8, 1937 | Anxiety neurosis | 0.326 | 81.5 |
| June 15, 1937 | Chronic rheumatoid arthritis | 1.15 | 83.0 |
| Jan. 12, 1937 | Erythematosus (lupus) | 0.914 | 80.1 |
| Dec. 11, 1936 | Arthritis (rheumatoid) | 1.10 | 80.0 |
| Average ascorbic acid content in the blood (The ascorbic acid saturated patient not included) | | 0.787 | |

*Before saturation. †Saturated before test.

TABLE III

RATE OF DISAPPEARANCE OF ASCORBIC ACID FROM THE BLOOD

GROUP III

Less Than 80 Per Cent Excretion

| DATE | DIAGNOSIS | ASCORBIC ACID IN BLOOD BEFORE TEST MG. PER 100 C.C. | PER CENT EXCRETION |
|---|---|---|---|
| Jan. 11, 1937 | Addison's disease | 0.367 | 79.4* |
| Feb. 17, 1937 | Syphilis—vaginal bleeding | 0.572 | 78.6 |
| Jan. 13, 1937 | Cerebral arteriosclerosis | 0.366 | 78.8 |
| March 24, 1937 | Fibromyoma of uterus | 0.76 | 78.0 |
| Dec. 2, 1936 | Migraine | 0.58 | 77.1 |
| March 24, 1937 | Irritability of the colon | 0.874 | 76.3 |
| Jan. 18, 1937 | Peptic ulcer | 0.431 | 76.0 |
| Dec. 9, 1936 | Arthritis (rheumatoidal) | 1.345 | 76.4 |
| March 2, 1937 | Peritonitis ? | 0.522 | 75.8 |
| Nov. 18, 1936 | Polycythemia | 0.59 | 75.6* |
| Nov. 30, 1936 | Polycythemia (same patient) | 0.808 | 64.2 |
| March 10, 1937 | Green streptococcus colitis | 0.418 | 75.7 |
| Dec. 17, 1936 | Diabetes mellitus | 1.70 | 75.0 |
| Jan. 29, 1937 | Addison's disease | 2.074 | 73.8† |
| Dec. 22, 1936 | Ichthyosis | 0.727 | 71.1 |
| Jan. 15, 1937 | Myocardial insufficiency | 0.545 | 67.6 |
| May 21, 1937 | Arteriosclerosis | 0.41 | 64.3 |
| Dec. 4, 1936 | Myxedema | 0.81 | 65.4 |
| Dec. 10, 1936 | Simmond's disease | 0.126 | 63.2 |
| Average ascorbic acid content in the blood (The ascorbic acid saturated patient not included) | | 0.664 | |

*Before saturation. †Saturated before test.

TABLE IV

URINARY EXCRETION OF ASCORBIC ACID FOR TWENTY-FOUR HOURS AFTER INJECTION OF 10 MG. SODIUM ASCORBATE PER KG. WEIGHT

GROUP I

30 per cent or more excretion

| DATE | DIAGNOSIS | ASCORBIC ACID IN BLOOD MG. PER 100 C.C. | PER CENT EXTRACTION IN URINE 3 HR. | 8 HR. | 24 HR. |
|---|---|---|---|---|---|
| Feb. 19, 1937 | Pernicious anemia | 1.544 | 37.9 | 45.6 | 84.3 |
| Feb. 11, 1937 | Anxiety neurosis | 1.610 | 33.5 | 46.7 | 84.2 |
| Feb. 11, 1937 | Allergic rhinitis | 0.924 | 41.6 | 56.9 | 60.3 |
| Jan. 15, 1937 | Addison's disease | 3.050 | 29.0 | 45.5 | 57.5* |
| March 30, 1937 | Kidney stone, streptococcus throat | 0.810 | 34.2 | 45.0 | 57.5 |
| April 28, 1937 | Erythematosis | 0.992 | 18.8 | 40.6 | 54.9 |
| March 4, 1937 | Asthma—morphine poisoning | 2.09 | 33.1 | 41.3 | 47.4 |
| Jan. 26, 1937 | Diabetic gangrene | 0.755 | 37.9 | 44.9 | 45.5 |
| June 16, 1937 | Chronic rheumatoid arthritis | 1.15 | 37.6 | 45.3 | 56.8 |
| March 10, 1937 | Green streptococcus colitis | 0.418 | 30.8 | 35.7 | 37.2 |
| March 18, 1937 | Intestinal neurosis | 0.744 | 32.6 | 35.2 | 37.6 |
| Dec. 22, 1936 | Ichthyosis | 0.727 | 16.4 | 23.8 | 34.0 |
| April 6, 1937 | Arteriosclerotic heart disease | 0.536 | 37.0 | 37.9 | 38.7 |
| Feb. 8, 1937 | Adenoma of bronchus (syphilis?) | 0.569 | 28.8 | 31.0 | 32.8 |
| March 12, 1937 | Cathartic colitis | 1.05 | 22.4 | 27.8 | 31.6 |
| June 6, 1937 | Diabetes mellitus | 0.73 | 25.1 | 30.0 | 30.7 |
| Nov. 24, 1936 | Diabetes mellitus | 1.06 | 25.5 | 45.6 | |
| Dec. 8, 1936 | Pernicious anemia | 1.236 | | 50.7 | |
| Dec. 10, 1936 | Simmonds' disease | 1.26 | 31.0 | 44.8 | |
| Dec. 9, 1936 | Rheumatoid arthritis | 1.345 | 31.3 | | |
| Average ascorbic acid content in the blood | | 1.045 ± 0.292 | | | |

*Saturated before test.

TABLE V

URINARY EXCRETION OF ASCORBIC ACID FOR TWENTY-FOUR HOURS AFTER INJECTION OF 10 MG. SODIUM ASCORBATE PER KG. WEIGHT

GROUP II

From 10 to 30 per cent excretion

| DATE | DIAGNOSIS | ASCORBIC ACID IN BLOOD MG. PER 100 C.C. | PER CENT EXCRETION IN URINE 3 HR. | 8 HR. | 24 HR. |
|---|---|---|---|---|---|
| Dec. 22, 1936 | Hyperthyroidism | 0.970 | 17.8 | 25.1 | 27.8 |
| March 24, 1937 | Irritability of the colon | 0.874 | 23.8 | 27.1 | 27.8 |
| Jan. 15, 1937 | Cerebral arteriosclerosis, myocardial insufficiency | 0.545 | 15.2 | 20.9 | 25.6 |
| Feb. 15, 1937 | Duodenal ulcer | 0.482 | 11.8 | 12.4 | 13.7 |
| Jan. 11, 1937 | Addison's disease | 0.367 | 17.6 | 23.1 | 24.5 |
| Jan. 27, 1937 | Mild diabetes | 0.423 | 22.1 | 24.6 | 26.7 |
| March 24, 1937 | Fibromyoma of uterus | 0.760 | 18.2 | 20.9 | 22.8 |
| April 6, 1937 | Rheumatic heart disease | 0.745 | 8.7 | 21.3 | 24.5 |
| Feb. 10, 1937 | Chronic cholecystitis | 0.480 | 19.1 | 19.9 | 21.0 |
| April 1, 1937 | Adrenal tumor | 1.320 | 16.8 | 17.4 | 18.2 |
| Feb. 17, 1937 | Vaginal bleeding—syphilis | 0.572 | 15.3 | 15.6 | 16.0 |
| March 4, 1937 | Right third nerve paralysis, gallstones | 0.475 | 12.9 | 13.2 | 15.8 |
| Feb. 2, 1937 | Cerebral arteriosclerosis | 1.750 | 10.8 | 11.6 | 13.8 |
| April 1, 1937 | Arthritis | 0.498 | 11.9 | 12.7 | 13.7 |
| Feb. 26, 1937 | Cardiac decompensation | 0.594 | 10.4 | 10.6 | 11.6 |
| Dec. 3, 1936 | Cholelithiasis | 0.780 | | 13.1 | |
| Dec. 2, 1936 | Migraine | 0.580 | | 24.1 | |
| Feb. 5, 1937 | Pulmonary tuberculosis | 0.332 | 9.2 | 9.5 | 12.0 |
| March 2, 1937 | Peritonitis ? | 0.522 | 10.7 | 12.0 | 12.8 |
| Average ascorbic acid in the blood | | 0.683 ± 0.256 | | | |

excreted about 30 per cent of the injected ascorbic acid in the first three hours after the injection, i.e., the largest percentage of the whole amount excreted during the twenty-four hours. Eight hours after the injection the total output was increased by only 10 per cent, to 40 per cent. As a rule, the individuals belonging to this group had a high ascorbic acid content in the blood before the injection (1.045 mg. per 100 c.c. ± 0.292). We may consider this group a normal one as far as the rate of urinary excretion of ascorbic acid is concerned. Those belonging to the second group excreted about 14 per cent in the first three hours; eight hours later, the ascorbic acid output rose by only 4 per cent, to 18 per cent; the ascorbic acid content in the blood of these patients was

TABLE VI

URINARY EXCRETION OF ASCORBIC ACID FOR TWENTY-FOUR HOURS AFTER INJECTION OF 10 MG. SODIUM ASCORBATE PER KG. WEIGHT

GROUP III

10 per cent excretion or less

| DATE | DIAGNOSIS | ASCORBIC ACID IN BLOOD MG. PER 100 C.C. | PER CENT EXCRETION IN URINE 3 HR. | 8 HR. | 24 HR. |
|---|---|---|---|---|---|
| June 4, 1937 | Subclinical scurvy | 0.500 | 7.9 | 8.5 | 10.3 |
| March 3, 1937 | Diabetes mellitus | 0.496 | 7.6 | 8.5 | 10.0 |
| May 19, 1937 | Polycythemia | 0.380 | 8.2 | 9.2 | 10.1 |
| April 9, 1937 | Malta fever (liver cirrhosis) | 0.375 | 5.2 | 6.5 | 9.1 |
| Jan. 13, 1937 | Cerebral arteriosclerosis | 0.366 | 6.5 | 6.6 | 9.5 |
| May 21, 1937 | Arteriosclerosis | 0.410 | 3.7 | 3.9 | 8.2 |
| Nov. 20, 1936 | Functional colitis | 0.386 | 6.9 | 7.3 | 7.7 |
| June 18, 1937 | Polyneuritis | 0.711 | 5.3 | 6.3 | 7.8 |
| Jan. 26, 1937 | Pernicious anemia | 0.765 | 2.8 | 7.1 | 7.5 |
| Feb. 4, 1937 | Ulcerative colitis | 0.377 | 2.0 | 5.1 | 7.0 |
| April 7, 1937 | Carcinoma of the stomach | 0.375 | 5.4 | 6.4 | 6.9 |
| Jan. 11, 1937 | Addison's disease | 0.412 | 2.7 | 2.9 | 5.8 |
| Jan. 15, 1937 | Same patient four days later | 0.356 | 0.6 | 6.1 | 6.6 |
| March 2, 1937 | Rheumatoid arthritis | 0.332 | 4.4 | 5.2 | 6.1 |
| Jan. 25, 1937 | Diabetes | 0.450 | 2.3 | 4.2 | 6.0 |
| March 5, 1937 | Neurosis | 0.332 | 3.6 | 4.2 | 5.5 |
| March 3, 1937 | Peptic ulcer | 0.698 | 3.3 | 3.7 | 5.1 |
| July 12, 1937 | Cirrhosis of the liver | 0.417 | 1.6 | 2.4 | 3.9 |
| Sept. 24, 1937 | Alcoholic polyneuritis | 0.265 | 1.2 | 1.6 | 3.6 |
| Jan. 12, 1937 | Nonspecific ulcerative colitis | 0.412 | 1.2 | 2.1 | 3.3 |
| Sept. 24, 1937 | Chronic nonspecific ulcerative colitis | 0.240 | 0.5 | 0.9 | 2.3 |
| Feb. 10, 1937 | Anxiety state | 0.297 | 4.9 | 5.5 | 6.5 |
| Nov. 25, 1936 | Stenosing duodenal ulcer | 0.394 | 6.5 | 7.4 | |
| Dec. 4, 1936 | Myxedema | 0.410 | 3.8 | 5.9 | |
| | Average ascorbic acid in the blood | 0.465 ± 0.104 | | | |

lower than that of the first group, 0.683 mg. per 100 c.c. ± 0.256. This group may be considered the border line between the normal and those cases having a definite deficiency. The patients belonging to the third group excreted only 4 per cent in the first three hours; eight hours later the urinary excretion rose by only 1 per cent, to 5 per cent; the average ascorbic acid content of the blood was also low, 0.465 mg. per 100 c.c. ± 0.104. Undoubtedly this group of patients may be considered as having a deficiency of ascorbic acid because 90 per cent or more of the injected ascorbic acid was retained by the tissues.

*Relation Between Ascorbic Acid in the Blood and Urinary Excretion.*—In spite of apparent discrepancies there seems to be a relation between the ascorbic

content in the blood and the per cent of urinary excretion in twenty-four hours after the injection of ascorbic acid, as can be seen in Fig. 3, where all the cases studied (63) have been plotted. The three cases having a high ascorbic acid content in the blood and a low urinary excretion deserve mention, because all of them had renal impairment. In the first case (acute morphine poisoning

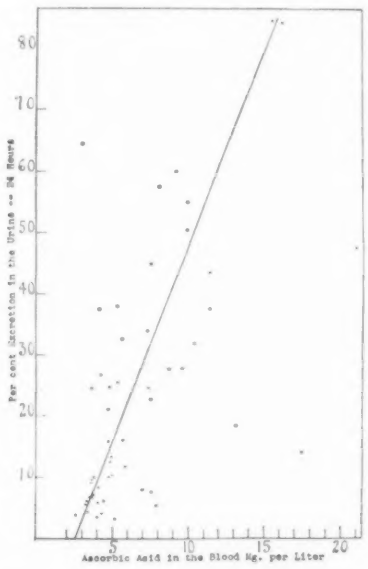


Fig. 3.—The relation between the ascorbic acid in the blood before the injection, and the urinary excretion, twenty-four hours after the injection of 10 mg. sodium ascorbate per kg. weight.

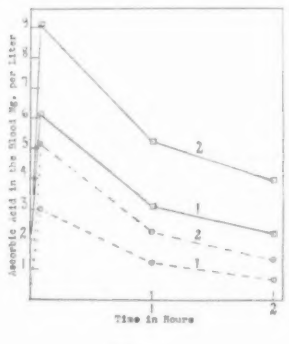


Fig. 4.

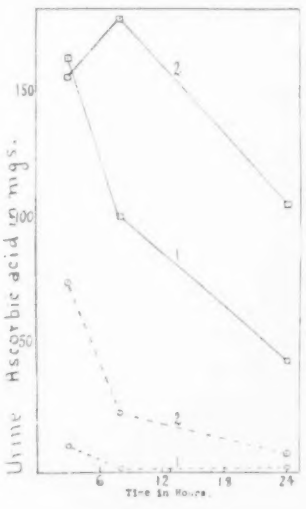


Fig. 5.

Fig. 4.—The rate of ascorbic acid disappearance from the blood after the injection of 10 mg. per kg. weight in two patients with Addison's disease. (1) Before saturation; (2) after the patients were saturated with ascorbic acid.

Fig. 5.—The rate of ascorbic acid excretion by the kidneys after the injection of 10 mg. sodium ascorbate per kg. weight in two patients with Addison's disease. (1) Before saturation; (2) after saturation.

followed by death some days later) the ascorbic acid content of the blood was 2.09 mg. per 100 c.c., and the urinary excretion only 47.4 per cent. In the second case, the blood ascorbic acid was 1.75 mg. per 100 c.c., with a urinary excretion of 13.8 per cent; the patient had an arteriosclerotic heart disease associated with cardiac hypertrophy, the urea clearance being 26 per cent below the mean normal. The third case, with a blood ascorbic acid of 1.32 mg. per 100 c.c. and a urinary excretion of 18.2 per cent was that of an adrenal tumor.

*Ascorbic Acid Excretion in Addison's Disease.*—Siwe,[12] from the study of one case, concluded that in Addison's disease there was a delayed excretion of added ascorbic acid. In Figs. 4 and 5, we give the excretion of ascorbic acid in two cases of Addison's disease before and after saturation. In one of these cases there was an obvious vitamin C deficiency in the body as shown by the low urinary excretion (5.8 per cent in twenty-four hours); in the other, excretion was 24.4 per cent. Three days after a daily injection of 10 mg. sodium ascorbate per kilogram weight, both cases became saturated and the blood ascorbic acid and urinary excretion rose accordingly. In both cases (one of them having an ascorbic acid deficiency) saturation was effected as rapidly as in all the patients with low ascorbic acid in the blood. If ascorbic acid deficiency is observed in Addison's disease, it must be a consequence of low vitamin intake or impaired renal function.

## DISCUSSION

A comprehensive discussion of the influence of ascorbic acid in the development of pathologic conditions other than scurvy is not possible without further elucidation of its physiologic functions. However, we may state with reasonable certainty that pathologic conditions will be produced only through deficiency, never through accumulation, because ascorbic acid, like all water-soluble vitamins, diffuses rapidly through the cellular membranes and, as a consequence, is quickly excreted by the excretory glands (kidneys, sweat glands).

Ascorbic acid, because of its being a reversible oxidation-reduction system, has been considered a catalyst for cellular respiration (Harris,[13] King[14]). So far no experimental proof has been presented to support this contention. Furthermore, from unpublished experiments performed in our laboratory, we postulate that ascorbic acid does not form part of the series of catalysts acting in cellular respiration. Ascorbic acid, a sluggish oxidation-reduction system, protected in the body from oxidation by the ordinary oxidation catalysts, seems to act as a promoter or catalyst of synthetic reactions (reductions, polymerizations), thus taking part in the building up of cellular and intercellular structures. This opinion is supported: (1) By studies on the pathology of scurvy (Wolbach and Howe,[15] Menkin, Wolbach, and Menkin[16]), where there is found a cessation in the normal formation of intercellular structures. (2) By its function of maintaining a normal level of blood hemoglobin, as shown by the decreased hemoglobin content in early stages of scurvy, which comes back to normal on administration of ascorbic acid before the clinical symptoms have subsided (Presnell,[17] Dunlop, Scarborough[18] and Kreitmair[19]); and by the prevention of cobalt polycythemia in rabbits when ascorbic acid and cobalt are given simultaneously (Barron and Barron[20]). (3) By its role in the formation of gly-

cogen as shown by a diminished liver glycogen in scorbutic guinea pigs which comes to normal on administration of ascorbic acid (Altenburger[21]), and by a decreased glucose tolerance curve (Sigal and King[22]) which we may interpret as a defective glycogen synthesis. (4) By its protective power against the oxidation of adrenaline and its probable role as a catalyst for the formation of adrenaline in the adrenal glands (Bourne[23]). (The protective power is easily understood because the oxidation-reduction potential at pH 7.0 of adrenaline is –0.389 volt, while that of ascorbic acid is –0.066 volt.)

A number of pathologic conditions with varied symptoms will, therefore, be found in the zone of ascorbic acid deficiency before scurvy is produced. Whenever the urinary excretion is increased, as might happen in certain cases of nephritis, in certain infections, and in conditions producing excessive sweat, there may develop an ascorbic acid deficiency, even though the vitamin intake is kept normal. Such conditions have been reported in pneumonia (Schroeder,[24] Bullowa, Rothstein, Ratish, and Harde[25]), in experimental nephritis (Pinotti[26]), in tuberculosis (Heise and Martin[27]), and in the mine workers in South Africa (Bernstein[28]). A similar condition will develop whenever the ascorbic acid intake is restricted, a condition frequently found in gastrointestinal disorders, e.g., in gastric ulcers (Lazarus,[29] Archer and Graham[30]), or in cases, still undetermined, where ascorbic acid is used up at a rate greater than normal.

Among the patients showing a definite vitamin C deficiency (ascorbic acid excretion, 10 per cent or less in twenty-four hours) there were found a miscellaneous group of diseases with no common syndrome except the ascorbic acid deficiency, probably produced by diet low in vitamin C content. Special attention must be drawn to the gastrointestinal cases, where the lowest urinary outputs were found. (One case of chronic nonspecific ulcerative colitis excreted only 2.3 per cent in twenty-four hours.) It is quite possible that many symptoms observed in gastrointestinal cases and catalogued as *functional*, or *neurotic*, are the consequence of hypovitaminosis due to the use of diets poor in vitamin content.

The determination of ascorbic acid in the blood and urine, accompanied by the twenty-four-hour urinary excretion after the intravenous injection of 10 mg. of sodium ascorbate per kg. weight, will allow us to detect varying degrees of vitamin C deficiency.

## SUMMARY

The rate of disappearance from the blood and the urinary excretion of ascorbic acid after the injection of 10 mg. sodium ascorbate per kg. weight was determined in 63 patients. A method is described for this study. The addition of 8-hydroxyquinoline and toluol to acidified urine allows the collection of twenty-four-hour urine specimens with no loss of ascorbic acid.

There seems to be, in general, a relation between the ascorbic acid content in the blood before the injection and the per cent of urinary excretion twenty-four hours after the injection; a urinary excretion of ascorbic acid of 10 per cent or less in twenty-four hours is evidence of vitamin C deficiency. Patients having less than 0.4 mg. per 100 c.c. of ascorbic acid in the blood showed, as a rule, a low rate of urinary excretion. Contrary to published reports there was

found no relation between the ascorbic acid content of the blood and the rate of its disappearance from the blood after the injection.

A survey of the physiologic functions of ascorbic acid suggests that its deficiency may produce varied pathologic alterations other than scurvy.

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# NOTES ON THE NATURE AND USES OF PROTAMINES IN THE TREATMENT OF DIABETES*

## Experimental Study of the Effect of Protamine Insulinate on the Estrous Cycle of White Rats

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PREVIOUS to their use in the treatment of diabetes mellitus the protamines were substances relatively unknown to the practicing physician. Indeed, many important textbooks on biochemistry and nutrition make no mention of them. Hence a brief statement as to their chemistry and significance may be of interest.[1, 2] They are simple proteins; that is to say, substances which yield only alpha-amino acids or their derivatives on hydrolysis. These proteins are classified chiefly according to their solubility, reaction to heat, and amino acid content. Included in the group are the albumins, globulins, albuminoids, histones, and protamines. The protamines are simpler polypeptides than the others of the group. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties, and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic amino acids greatly predominate.[3] The protamines occur in combination with nucleic acids in spermatozoa. In studies thus far made, fish have been the chief sources; thus salmine is obtained from salmon, sturine from sturgeon, scombrine from mackerel, cyprinine from carp, and clupeine from herring. The protamines are divided into three groups, the mono-, di-, and tri-protamines, according to their content of one, two, or three basic constituents, lysin, arginine, and histidine.

Their use depends upon the following principle. They combine with insulin, an acid sulfur-bearing protein, to form a salt which is but slightly soluble in the tissue fluids. The minimum solubility or isoelectric point of insulin is approximately at pH 5.3. The solubility increases above and below this concentration level. Salmine, the protamine from the Columbia River salmon, *Salmo iridus*, has a pH of 12. When combined to form protamine insulinate (Lilly), its pH varies from 7.1 to 7.3, which is its point of least solubility. As it is buffered to the pH 7.4 of the tissue fluids when injected into the body, its solubility slightly increases. Tissue juices dissolve it more rapidly than do watery solutions. Therefore, when a pool of protamine insulinate is injected under the skin most of the salt is in suspension, a very small amount

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being available for absorption immediately. In 1 c.c. of protamine insulinate (Lilly), as injected into the skin, only about 0.2 unit is in solution and available for immediate absorption. With 1 c.c. of regular insulin (U-40) the entire 40 units are readily absorbable. The amount of insulinate in solution is independent of the total concentration of the suspended material; that is to say, the amount is the same whether U-20, U-40, or U-80 insulinate is used. As absorption takes place the fluid of the insulinate is replaced by tissue juices and solution then takes place at an accelerated rate. This delayed solution of the protamine material serves to prolong the physiologic action of the insulin, which is the effect desired. Absorption which, with regular insulin, takes place in about twenty minutes, with protamine insulinate is thus delayed for many hours.

In the pre-insulin era of diabetic therapy menstrual disorders were both common and severe. Many patients would go for months or years without menstruating. Lack of fertility was likewise startlingly evident. The advent of insulin and the liberal general diet which this made possible, has completely changed this condition and has brought about a more nearly normal sexual state. This has been the experience of practically all workers in the field of diabetes. Nevertheless, it is recognized that there is a certain relationship between the estrogenic and the insulin functions. The implications of this problem are not clearly understood. Recently Collens and co-workers[4] reviewed the subject, cited some case reports, and concluded that estrogenic substance suppresses anterior pituitary function and that the administration of the estrogenic substance partially prevents the development of diabetes following pancreatectomy; that estrogenic substance does not have any beneficial effect on the tolerance of the diabetic patient. This is in accord with the clinical experience of the author.

Altschul,[5] in a study of dysmenorrhea in nulliparous, nondiabetic females, classified his patients into four groups, in one of which he believes the disturbance to be due to insulin deficiency. He proceeded to treat twelve such cases with varying doses of insulin, with considerable relief. It seems quite probable, therefore, that the conclusion is well founded that there is a definite relationship between the functions of menstruation and insulin activity. During the past year, in a study of the clinical effects of protamine insulinate on female diabetics, the question arose as to whether the protamine factor had any influence on the menstrual cycle. This question was precipitated by the fact that two young females had temporary interruptions of this function while using protamine insulinate. The fact, also, that the protamine used in the preparation of protamine insulinate is of gonadal origin adds interest to the query. Accordingly an experiment was undertaken to determine the effect of therapeutic doses of insulin and protamine insulinate on the estrus cycle of the white rat.

The effect of insulin injections alone on puberal rats was recently studied by Crainicianu and Capelman.[6] The authors reviewed previous work on this subject and experimented and observed the effect of insulin injections on the estrual rhythm of twenty puberal rats for a period of fifteen days. Their conclusion is that the hormonal action seems to be a natural excitor of the

female genital organs. It should be pointed out, however, that the dosage given most of their rats was far in excess of that used therapeutically on human patients.

It is recognized that the estrus of the rat does not exactly parallel physiologically the menstrual cycle of the human being. It is more sensitive than the latter, but the phenomena are closely related. For this reason the rat was used as a basis for experimentation. According to Long and Evans,[7] the estrus cycle of the rat normally lasts four days. It is characterized by a regular, periodic, coordinated, histologic change in every portion of the reproductive tract, including ovulation. These changes may be followed by making smears of the vaginal contents. Five different histologic pictures are thus presented, each representing some phase of the normal cycle.

For the purposes of our study twelve healthy albino female rats were selected, having a weight range of from 105 to 158 gm. Of these, five were used as controls, and seven for the various tests. They were first observed

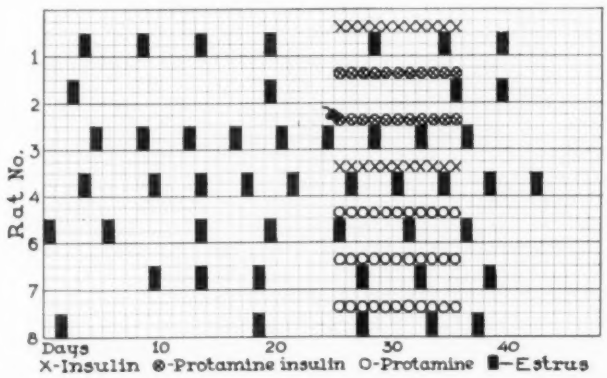


Fig. 1.—Chart illustrating the relation of insulin, protamine insulinate, and protamine to the estrus cycle of white rats. No significant change is noted.

as having a normal estrus cycle for a period of two weeks, to ensure their fitness for these tests. It was not the purpose of the experiment to determine the lethal effect of either the protamine or the insulin, but rather to ascertain if these materials used in amounts which would correspond to the therapeutic dose in the human being would have any effect on the estrus cycle. Two of the rats, after the preliminary period of observation, were given regular clinical insulin, 0.2 unit dissolved in 0.2 c.c. of Ringer's solution, daily over a period of eleven days. During this time there was no disturbance in the estrus cycle or in the body weight, beyond that of normal health. Likewise two other rats, after the period of preliminary observation, were given protamine insulinate in similar dosage over periods of eleven and fifteen days, respectively, without any alteration in the estrus cycle. Three of the rats, after the preliminary observation period, were given daily injections of the pure protamine solution of 0.01, 0.02, and 0.04 c.c., respectively, dissolved in 0.5 c.c. of Ringer's solution, over a period of eleven days. None of these rats showed any noteworthy change in the estrus cycle.

The conclusion to be drawn from this limited experiment on white rats is that neither protamine insulinate nor pure protamine, administered in therapeutic doses, has a noticeable effect on the estrus cycle.

The writer is indebted to Louise Huey, A.B., and to Professor George W. Corner, for advice and assistance in the conduct of the experiments.

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# EXPERIMENTAL AND CLINICAL CONSIDERATIONS OF THE ACTIONS OF CAMPHORTETRAZOL, METRAZOL, AND OF SCHIZOPHRENIC PSYCHOSES*

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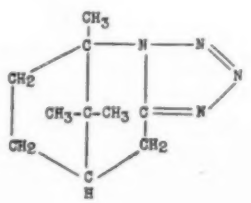
CAMPHORTETRAZOL is a white, crystalline substance with a melting point of 242° C., and a molecular weight of 192.2. It is a slightly soluble powder and for therapeutic use dissolves in a solution of urethane or 20 per cent alcohol. A 2 c.c. dose contains 40 mg. of camphortetrazol and $\frac{4}{10}$ gm. of urethane in sterile aqueous solution. But in the experiments here reported we have used 2 per cent solution of camphortetrazol in 28.5 per cent alcohol. In order to insure against slow precipitation of the drug from solution it is necessary to use approximately this percentage of alcohol.

Camphortetrazol is methylisopropylidenpentamethylentetrazol—$C_{10}H_{16}N_4$—having the following graphic formula:

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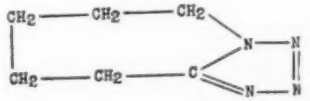
It thus is related chemically to metrazol which is pentamethylentetrazol—$(CH_2)_5CN_4$—and which has the following graphic formula:



Metrazol is a white, crystalline, faintly bitter and practically odorless powder, with a melting point of 58° to 59° C., molecular weight 138. It is freely soluble in water, up to a 50 per cent solution being possible. Solutions are neutral, stable to light and heat, and may be sterilized by heat. It is marketed in ampoules in 10 per cent neutral aqueous solution, each ampoule containing 1½ grains of the drug. Both metrazol and camphortetrazol are sold in this country by the Bilhuber-Knoll Corporation, Jersey City, N. J.

In the following experiments we have compared the relative actions of camphortetrazol and metrazol as we believe this will be useful to other workers. It is to be noted that the solutions of camphortetrazol used are of 2 per cent strength, while those of metrazol are 10 per cent, five times as strong, but pharmacologically camphortetrazol is much more powerful than is metrazol, and hence smaller doses should be used. We have not yet had an opportunity to administer camphortetrazol to human beings, but we have examined its action on dogs, and by comparison of the results obtained with those produced by metrazol and other drugs, we have arrived at certain conclusions regarding the probable usefulness of camphortetrazol.

Camphortetrazol is a convulsant drug which can produce both clonic and tonic convulsions. The medulla is directly stimulated and the action of the drug extends both upward into the brain stem and, in lesser degree, to the cortical motor areas, and downward into the spinal cord. The convulsions thus are rather more of the clonic (medullary) type than of the tonic (spinal cord) or epileptiform (cerebrum types), although during marked convulsive seizures the consciousness is seriously disturbed if not entirely lost.

It is our opinion that the chief clinical interest in camphortetrazol will lie in one or more of three directions. These are, first, its action on the respiratory center, second, its possible emergency improvements in the circulation, and third, its use in the treatment of schizophrenia. While we unfortunately have not been so situated as to be able to fully investigate this latter suggestion, still it is our opinion that camphortetrazol will most likely find its greatest field of usefulness in the treatment of the ill-defined mental states now generally classified under the name of schizophrenia.

When 0.5 c.c. of 2 per cent camphortetrazol (in 28.5 per cent alcohol) is injected intravenously into an 8 kg. dog, the animal will, in a few seconds, show marked generalized symptoms, depending somewhat on the rate of injection. The symptoms are more marked and more prolonged if 1 c.c. or more is injected at a dose. But we suspect on the experimental basis, counting the weight of the dog as against the weight of a patient, that 0.5 c.c. of the solution (2 per cent) produces about the same extent of convulsive seizures in the dog as would be of maximum therapeutic efficiency in a schizophrenic patient.

When the drug is injected, the dog may at first remain quiet but alert and apprehensive for about five seconds, and then a few beginning clonic convulsive movements may occur. Suddenly, much more marked convulsions, generalized in type but inclining rather to the clonic form, appear. Partial relaxation may

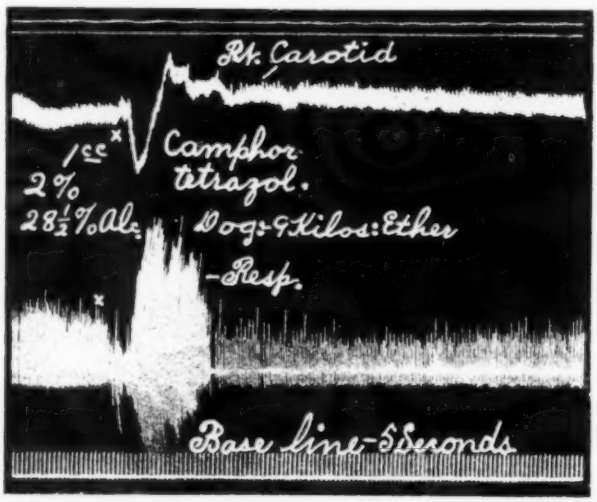



Fig. 1.—For discussion see text.

Fig. 2.—For discussion see text.

then follow, but immediately there occurs a profound generalized convulsion involving both tonic and clonic movements. The consciousness is seriously disturbed or lost altogether, the eyes roll about but with a tendency to lateral nystagmus, and the pupils constrict but from time to time may dilate. Repeated convulsive movements occur, decreasing in intensity for about three to four

minutes. A stage of apparent bewilderment with rapidly returning consciousness follows. A few isolated, clonic convulsive movements occur, the animal stretches its limbs, responds well to noises or signals, and in about four and one-half to six minutes stands up, and in a few seconds more may start to trot about the room or to play with the attendant. With a larger dose, or with other dogs, a more prolonged stage of recovery with some depression and lassitude extending over a period of fifteen to thirty minutes may occur. But we anticipate that the rapid recovery would be equally effective and more desirable from a clinical standpoint.

It will be noted that these symptoms are very similar to those produced by metrazol. It would be impossible to anticipate without extensive clinical trial which of these two drugs may be the more effective in schizophrenia. But of the general similarity of their actions there can be no doubt.

Fig. 1 shows the action of camphortetrazol on the blood pressure and respiration. The animal was etherized; it should be emphasized that both the

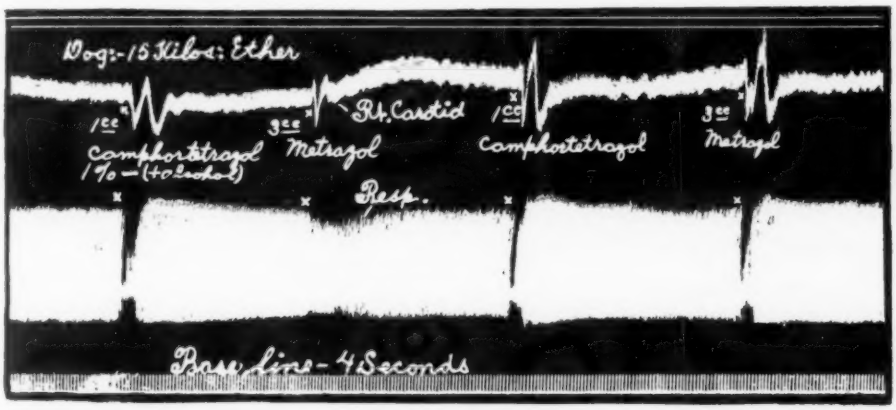



Fig. 3.—For discussion see text.

type of drug used to produce the anesthesia and the depth of the anesthesia have a profound influence on the action both of metrazol and of camphortetrazol. But of the two the camphor compound is much more active in producing stimulation of the central nervous system. Fig. 1 shows a marked temporary stimulation of respiration. But this is rather convulsive in nature and may not be of much value clinically, although the anesthetic here masks the respiratory stimulation a good deal.

Fig. 2 shows a rise in blood pressure and the development of convulsions. The latter shows in the irregularity of the respiratory tracing. But it is impossible from the respiratory tracing alone to demonstrate the extent and the full character of the convulsions in any given case. As one watches the movements, however, the impression is gained that the seizures are more marked in the shoulders, forelimbs, head and neck regions than is the case with the hindlimbs and caudal portions of the body. The anesthetic may have some influence on these effects.

Fig. 3 shows the comparative effects of two injections each of camphortetrazol and metrazol. Those who are familiar with the clinical action of large

intravenous injections of metrazol will see the striking similarity at once. But the actual quantity of metrazol injected (300 mg.) is, of course, much larger than is the case with the more active camphortetrazol. Convulsive movements follow each injection of each drug. But these are indicated only in a general way in both the blood pressure and the respiration.

Fig. 4 shows the results of three injections of camphortetrazol. Here much greater rises in blood pressure are produced than were shown in the preceding tracings. All of these blood pressure records were made with a mercury manometer, and in each case the ether anesthesia modified to a marked extent the general appearance of the blood pressure tracing. But in tracing No. 4 a marked and prolonged rise in blood pressure is produced at each injection.

Our experiments with camphortetrazol have led us to inquire into the nature of possible drug reactions on the central nervous system which might reasonably be expected to help a schizophrenic patient. A cursory survey of current hypotheses proposed to explain the action of such widely diffffering preparations as carbon dioxide, cyanides, insulin, amytal, metrazol, etc., is far

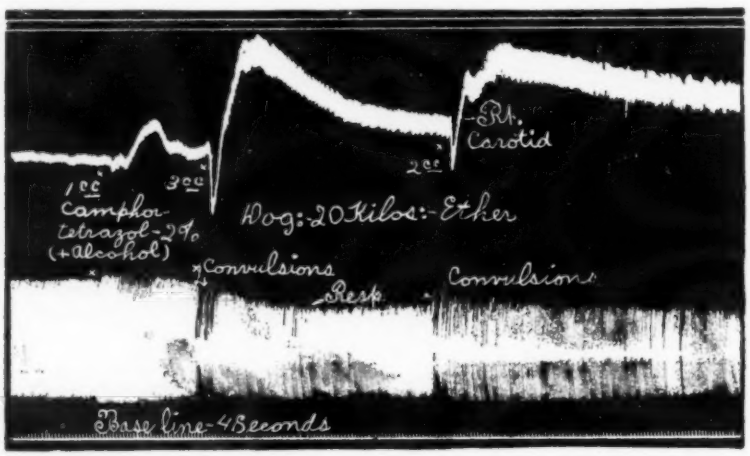



Fig. 4.—For discussion see text.

from reassuring, and we believe that practically all of these are entirely erroneous. Loevenhart[1] and his colleagues supposed that the oxidations in the brain cells were modified in some unknown fashion by small doses of cyanides and carbon dioxide, and that this served to brighten up the patient's mental state for a very brief period (two to twenty-five minutes). The idea implied that oxidations were obstructed in the brain cells and that this served to stimulate them. Hoskins,[2] as the result of recent observations, has concluded that defective oxygen metabolism is an important feature in the schizophrenic psychosis during the ordinary course of the disease.

In our opinion, such drugs as metrazol, camphortetrazol, cyanides, carbon dioxide, and insulin (shock) can have only a single feature in common in their actions by which schizophrenia (and probably various other mental abnormalities) can be permanently benefited. This feature consists of the sudden powerful rises in the systemic blood pressure which are produced by the acute convulsions. These rises, we believe, force open capillaries or other larger blood

vessels in the brain and thereby supply sufficient oxygen to the cerebral cells to enable them again to carry out more or less perfectly their normal functions which we believe have been depressed by some obstruction to the blood supply to the brain cells. There are obviously many points which need to be explained in order to fully establish this view. And it will take much time and many workers to completely cover this entire field. We make this suggestion now because we believe it will be helpful to those who are at present doing such splendid work with this large and ever increasing army of patients. We also suspect that the chills (*shivering*) produced by malaria are the real cause of any *mental* improvement shown by patients having general paresis who are treated by this method. In all cases if too great an organic deterioration of the brain cells has occurred before the treatment is begun, then complete restoration cannot be attained. The suggestion has already appeared in the literature that schizophrenia may be an aftermath of rheumatic or other acute infections. In our opinion a dilatation of the capillaries or arterioles and venules in the brain by sudden rises in systemic blood pressure produced by convulsions are of exactly the same character as is the dilatation of the vessels of the foot produced by suction and pressure devices of which a number have been in wide use in recent years for the treatment of gangrene and other vascular disturbances. There is, of course, a mechanical difference between these two methods of increasing the circulation, but the ultimate results should be exactly the same. While extra blood is sucked into the foot vessels which are thereby dilated (if the circumstances are favorable) by the suction apparatus, on the other hand, extra blood should be forced in the brain vessels which should thereby be dilated (if the circumstances are favorable) by the suddenly increased systemic blood pressure. If one asks, How can the blood supply to the foot or to the brain be increased? we have here a sort of double answer, the one a kind of reply valiant, the other a retort courteous.

The circumstances governing an increased blood supply to the brain as a result of a sudden rise in pressure in the systemic circulation are exceedingly complex. Under ordinary circumstances a marked rise in blood pressure will be actively opposed by reflexes originating in the carotid sinus and aortic arch. These reflexes lead to a slowing of the heart (vagus) and a dilatation of systemic arterioles. These results would follow a moderately rapid or a slow rise in blood pressure. But a sudden strong generalized convulsion, especially involving the abdominal muscles, would suddenly shoot the blood pressure up perhaps to a great height before the reflex protective mechanisms could have a chance to counteract the rise in pressure. By new and refined technique, Hamilton, Woodbury, and Harper[3] have recently shown that during coughing or straining, the systolic arterial pressure may suddenly shoot up to an enormous height. Under these circumstances pressure inside the skull would depend a good deal on the rate and extent of venous outflow through the jugular veins, and this in turn would depend largely on the intrathoracic pressure.

During convulsions the respiration becomes greatly disturbed, asphyxia and gasping movements occur, and these may both be further complicated by repeated spasms. Asphyxia causes a rise in blood pressure even independently of the convulsant action on the central nervous system.

It should be noted that the rises in blood pressure shown in Fig. 4 come on relatively slowly and that they continue for at least some minutes. These rises were recorded with a slowly moving mercury manometer, and we doubt that such rises in pressure as are seen here would be of much value toward causing a *permanent increase* in circulation through the brain vessels. But with a very sensitive and rapidly recording device, such as Hamilton, Woodbury, and Harper have used, we may expect to see many sharp, sudden rises and falls of pressure superimposed on the mercury manometer tracing, these rapid vacillations depending on the individual spasms, clonic or tonic or both intermixed. It is these sudden short upshoots of pressure that we suspect may cause an effective opening up of the brain vessels. As evidence of this we may recall that a number of patients who have died during treatments for schizophrenia have been shown at autopsy to have suffered more or less severe brain hemorrhages. During insulin shock many patients have hemiplegia, facial or otherwise localized paralyses or paresthesias. These usually clear up rather rapidly unless a more severe brain hemorrhage has occurred.

It does not seem probable to us that there is anything *specific* in the action of any of the drugs used in the treatment of these cases. It would appear rather that such beneficial results as may occur are due solely to accidental side actions which these and perhaps a number of other drugs may produce. In this connection it would seem probable that a drug which would produce convulsions quickly, of any desired severity, and whose action could be quickly and completely controlled, both by the dose and by antidotes would be the best type of compound to use. Metrazol comes near to meeting these requirements. We believe that camphortetrazol will also fall into this group, but whether it will be equal to or better than metrazol we cannot now state.

The actions of insulin are obscure and we are of the opinion that the changes in sugar metabolism produced by it are not beneficial to the patient, but are rather the reverse, except that the low blood sugar in some way contributes to or produces the convulsions. Recent work by Hrubetz[4] rather suggests that insulin may have some specific action on the medullary centers independent of the blood sugar changes. But this problem is difficult to investigate. Barbiturates will prevent insulin convulsions. Hrubetz administered barbiturates and large doses of insulin to rabbits. In no case were there any signs either of convulsions, or even of the prodromal signs of insulin action on the central nervous system. Although no glucose was administered, none of these animals died despite the fact that the true blood sugar levels remained at or near the zero level for at least the three-hour observation period. Apparently, the protective mechanism of the barbiturate is independent of the blood sugar concentration.

It has been shown by Comroe and Addison[5] that the hypertension of anoxia is produced by reflexes arising chiefly in the aortic, and to a lesser extent in the carotid, chemoreceptors. The latter exceptionally produce marked reflex hypertension, but only if the opposing reflex bradycardia is eliminated by vagotomy. When large doses of a drug like metrazol are injected intravenously, there may be some action produced by the drug through its stimulation (or irritation) of the aortic or carotid bodies.

We suspect that such temporary brightening up of the schizophrenic's mentality as may occur from the inhalation of carbon dioxide in oxygen is due to a

stimulation of the same physiologic mechanism as is brought into play by metrazol or insulin, and possibly carbon dioxide inhalations might be used as a sort of preliminary test to indicate whether or not any given patient would probably be (or not be) benefited by the much more heroic and expensive treatment with insulin or metrazol.

Fig. 5 shows well the comparative actions of camphortetrazol and metrazol. Without labels it would be difficult to distinguish between these tracings. When nonanesthetized dogs are given intravenous injections of either of these drugs, if the dosage is correct, recovery occurs in a few minutes and is apparently complete and permanent. We have even noted occasionally that when a dog is injected with a moderate sized dose of camphortetrazol the animal may, after recovery, show what appears to be a distinct increase in alertness and intelligence for fifteen or twenty minutes.

There might be some question about injecting a 28.5 per cent solution of alcohol intravenously. Undoubtedly this is undesirable as there might be some possibility of flocculation or precipitation of the blood. In our experiments,

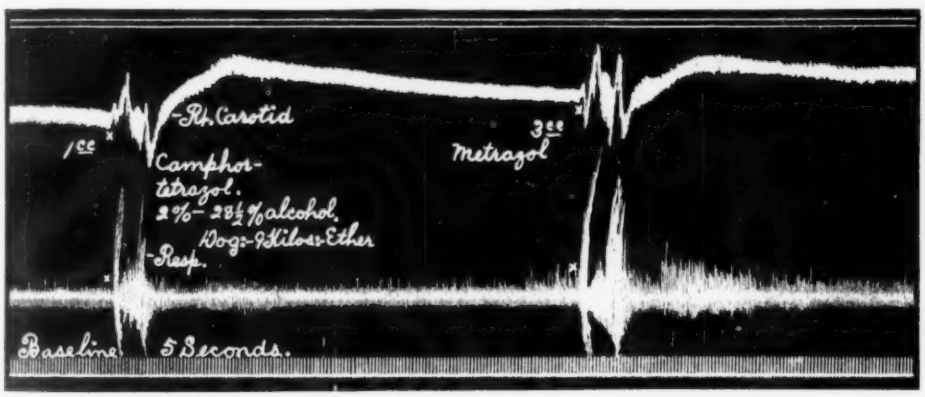



Fig. 5.—For discussion see text.

however, we have seen no deleterious results which could be attributed to this cause. It may be possible that some more satisfactory means of dissolving camphortetrazol can be found.

It is interesting to note in connection with the older work on the use of cyanides to stimulate cerebral action that Schmidt,[6] Comroe[7] and their colleagues and others have shown that marked vasopressor reflexes are produced quickly by cyanides through chemical stimulation of receptors in the carotid and aortic bodies, more especially in the latter. Carbon dioxide and anoxia play similar roles. Earlier workers in these fields were, of course, unfamiliar with the nature of these reflexes.

It seems hardly necessary to suggest that *permanent changes* produced in the mental state of a patient by drugs, which when administered are quickly destroyed or excreted from the body, must appear to be due to the development of some *permanent* physiologic or anatomic change in the brain.

We are not unmindful of the fact that some workers may suggest that such drugs as metrazol, insulin, cyanides, or carbon dioxide act by producing some sort of psychical or spiritual shock, and that this is the thing which helps or

cures the patient. In these cases we suspect either that some revision in the matter of diagnosis should be made in those particular instances, or else that some very much more definite and clear-cut understanding of the nature of the mental states and reactions existing in schizophrenia, so called, and of the anatomic, physiologic and pathologic bases on which these states and reactions depend, should be introduced into psychiatry. For we are as yet hardly in a position to discuss profitably "spiritual pharmacology."

## CONCLUSIONS

1. The pharmacological action of camphortetrazol is discussed.

2. The action of metrazol is compared with that of camphortetrazol. The clinical application of metrazol, insulin, carbon dioxide, and cyanides in schizophrenic psychoses is considered in relation to the pharmacological action of camphortetrazol.

3. Certain physiological phenomena and reflexes are discussed with reference to the therapeutic treatment of schizophrenic patients.

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# BIOCHEMICAL AND MORPHOLOGIC METHODS FOR THE ISOLATION AND IDENTIFICATION OF YEASTLIKE FUNGI*

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CLASSIFICATION of yeastlike fungi based on biochemical, morphologic, and serologic properties has long presented difficulties. Species have been inadequately described, and morphologic and biochemical variations reported have contributed to this disorder and to a confused nomenclature. The genus Monilia, as recognized by medical mycologists, has received most attention. Fisher and Arnold (1936) presented a thorough review of early studies.

*From the Department of Bacteriology, University of Maryland, College Park.

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Recently several investigators have introduced methods of identification worthy of consideration. Benham (1931) emphasized morphology of the mycelium as observed in corn meal agar cultures, and also used giant colony characteristics, carbohydrate fermentations, agglutinin absorption, and pathogenicity tests, in studying representative yeasts and yeastlike fungi.

Stovall and Bubolz (1932, 1933) classified over 250 cultures of Monilia and yeastlike fungi isolated from sputa, from various infections and collected from other workers and the American Type Culture Collection into three types: *M. parapsilosis*, *M. albicans*, and *M. candida*. They differentiated these on fermentation of maltose and saccharose, on forty-eight-hour colony mycelium production on malt agar, and on coagulation of milk that was sterilized by Arnoldization in 10 c.c. quantities, and to which was added 0.5 c.c. of sterile 10 per cent calcium lactate solution.

Fisher and Arnold (1936) grouped yeasts and yeastlike fungi into Saccharomyces, Cryptococcus, Monilia, and Endomyces. The genus Mycoderma was not placed in the group of yeastlike fungi. Four species of Monilia were recognized, namely, *M. albicans*, *M. parapsilosis*, *M. candida*, and *M. krusei*, and occasionally types which differed from these species were encountered. The authors stated that a study of the morphology of the species (using maltose broth, corn meal agar, giant colonies, and ascospores from old dried agar slant growth) coupled with a study of biochemical reactions (form of growth in gelatin, milk coagulation, and sugar fermentation) should make identification simple.

Martin and others (1937) divided Monilia into six species, the four described by Fisher and Arnold, together with *M. mortifera* and *M. stellatoidea*. A special technique of identification was used, whereby the fungus was isolated on Sabouraud's glucose agar, transferred to Sabouraud's glucose acid broth, streaked on a beef extract blood agar plate, and then transplanted to a Sabouraud's glucose agar slant. A carrot plug was streaked from this for determination of ascospores. A beef extract agar slant was streaked and the growth subcultured on this medium for two or three generations, then a corn meal agar slide culture and four sugar broths, namely, glucose, saccharose, lactose, and maltose, were inoculated.

## EXPERIMENTAL PROCEDURE

In the selection of media and methods of classification to be used, particular attention was given to simplification of technique and to the use of standard procedures. Thirteen cultures of yeastlike fungi isolated from pathologic conditions* were employed.

*Comparison of Media.*—Four solid media commonly used for growing yeastlike fungi were prepared, these containing the following ingredients per liter of tap water (the pH of these media being unadjusted):

Malt agar—Difco malt extract 30 gm., Bacto agar 15 gm.

Honey agar—honey 60 gm., peptone 10 gm., Bacto agar 15 gm.

Sabouraud's maltose or dextrose agar—maltose or dextrose 40 gm., peptone 10 gm., Bacto agar 15 gm.

*We are indebted to Miss Helen Spicer of the Washington Sanitarium and Hospital, Takoma Park, Md., for eleven of these cultures.

Suspensions of each of ten cultures of yeastlike fungi from pathologic conditions were plated upon these four media. The results from two series of platings are presented in Table I, plates from the same dilution being compared for each culture. Malt agar gave consistently high counts and larger colonies compared with the other three media. The fungi produced heaviest growth on malt agar slants, and in other determinations when seeded on plates in numbers too large to count, they produced the greatest number of colonies on malt agar, as determined from appearance. Since the malt agar was also

TABLE I

COMPARATIVE COLONY COUNTS OF YEASTLIKE FUNGI ON COMMONLY USED AGARS

| CULTURE NO. | MALT | HONEY | SABOURAUD'S | |
|---|---|---|---|---|
| | | | MALTOSE | DEXTROSE |
| Series I | | | | |
| 1* | 1800 | 1850 | 1450 | 1750 |
| 2* | 2000 | 1590 | 1520 | 1530 |
| 3a* | 2600 | 1900 | 1900 | 1650 |
| 4 | 11 | 14 | 13 | 12 |
| 6 | 37 | 28 | 36 | 28 |
| 7 | 30 | 29 | 23 | 28 |
| 8 | 28 | 24 | 31 | 17 |
| 9 | 26 | 19 | 36 | 29 |
| 14 | 32 | 26 | 30 | 29 |
| 15 | 69 | 87 | 93 | 86 |
| Series II | | | | |
| 1 | 28 | 28 | 26 | 27 |
| 2 | 47 | 41 | 56 | 35 |
| 3a | 62 | 56 | 55 | 48 |
| 4 | 18 | 10 | 19 | 19 |
| 6 | 17 | 30 | 33 | 25 |
| 7 | 28 | 28 | 25 | 31 |
| 8 | 17 | 25 | 15 | 14 |
| 9 | 12 | 19 | 24 | 25 |
| 14 | 21 | 20 | 24 | 27 |
| 15 | 20 | 19 | 15 | 23 |

*Estimated from portion of plate counted.

easily prepared, it was used throughout this work, although it did contain a precipitate which was found unobjectionable. The agar was acidified before use, in order to eliminate bacterial growth, by the addition of 1 c.c. of a $\frac{1}{10}$ dilution of lactic acid, C. P., to 100 c.c. of melted agar, yielding a pH of approximately 4.0.

*Studies on Enrichment Broth.*—Honey broth was used in sampling the normal fingers for yeastlike fungi. Since enrichment might increase the number of isolations positive for yeastlike fungi, experiments were performed using a culture later identified as, or closely related to, *Monilia parapsilosis*. Inoculations were made into broth adjusted with N/1 NaOH and a $\frac{1}{10}$ dilution of lactic acid. Amounts sufficient to give a *final* pH ranging from 4.1 to 5.8 were used, since autoclaving lowered the pH greatly, especially above pH 5.0. A finger was dipped into each jar containing 5 c.c. of honey broth before inoculation with the yeast culture, in order to furnish bacterial competition. Each jar was inoculated with a wire loopful of a suspension of yeast cells of strain 16 (the number of viable yeast cells being determined by plating one loop of the yeast dilution). After enrichment by incubation for varying times at room

temperatures and at 37° C., the numbers of cells in each jar of inoculated honey broth was determined by plating. The results of these studies are shown in Table II. Honey broth of pH 5.4 to 5.8, incubated at room temperature for twenty-four hours before plating with malt agar, was more favorable to the multiplication of yeast than broths of lower pH incubated for shorter times at room temperature, or at 37° C. For further isolations, broths of pH 5.0 to 5.4 were employed. A higher pH might have been more favorable to yeast growth, but likewise would have favored bacterial multiplication.

*Fermentation of Carbohydrates.*—The value of fermentation reactions in the identification of yeastlike fungi has been debated. Investigators have reported fermentive variability between duplicate tubes and tests run at dif-

TABLE II

EFFECT OF pH AND INCUBATION UPON THE VIABILITY OF A MONILIA SPECIES INOCULATED INTO HONEY BROTH

| INCUBATION | | PLATE COUNTS PER C.C. AT pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SERIES I* | | | | | SERIES II† | | |
| TIME | TEMPERATURE | 4.1 | 4.3 | 4.8 | 5.0 | 5.4 | 4.4 | 5.0 | 5.8 |
| 0 | | 12 | 12 | 12 | 12 | 12 | 6 | 6 | 6 |
| Two minutes | Room | | | | | | 5 | 2.5 | 4.5 |
| Fifteen minutes | Room | 9 | 10 | 9 | 7 | 6 | | | |
| One and one-half hours | Room | 7 | 5 | 8 | 16 | 14 | 0 | 0.5 | 0 |
| One and one-half hours | 37° C. | 9 | 9 | 5 | 6 | 4 | 1.5 | 0 | 1 |
| Twenty-four hours | Room | 240 | 330 | 370 | 568 | 1150 | 149 | 118 | 311 |

*Single plates.
†Average of duplicate plates.

ferent times. Stovall and Bubolz (1932), however, reported constant reactions over a period of seven years. Hopkins and Hesseltine (1936) stated that the amount of inoculum affected the fermentation reactions, a large inoculum causing fermentation in some cases, whereas a small inoculum did not. Martin and associates (1937) cultured Monilia species on nutrient agar for several generations before inoculating fermentation tubes which were sealed with vaseline, and they obtained consistent reactions.

Before selecting a basal broth for the carbohydrate tests, different broths including those used by previous investigators were compared, using fermentation results produced by eleven yeastlike fungi from diseased persons. The broths contained the following ingredients per liter of tap water:

1. Stovall and Bubolz (1932)—3.0 gm. beef extract, 5.0 gm. peptone.
2. Hopkins and Hesseltine (1936)—3.5 gm. beef extract, 10 gm. peptone, 5.0 gm. NaCl.
3. Kurotchkin (1934)—5.0 gm. peptone, 1.0 gm. NaCl.
4. Ashford (1932)—10 gm. peptone.
5. Dunham's peptone (Benham, 1931)—10 gm. peptone, 5.0 gm. NaCl.
6. Yeast extract—2.5 gm. yeast extract, 5.0 gm. peptone.

These broths were prepared incorporating 1 c.c. of a 1.6 per cent alcoholic solution of bromthymol blue and bromcresol purple indicators, per liter of broth adjusted to pH 7.2. Broths were dispensed in 9.5 c.c. quantities in 6

by ¾ inch tubes containing an inverted 3 by ¼ inch tube to collect gas. Carbohydrates were sterilized in 10 per cent distilled water solutions in an autoclave for ten minutes at 15 pounds pressure, and 1 c.c. added aseptically to each broth tube, which was incubated at 37° C. for twenty-four hours to check sterility. Duplicate tubes were inoculated with a portion of a loopful of forty-eight-hour yeast culture, care being taken to add as constant quantities of culture as possible, and to disperse the inoculum in the fermentation broths. Saccharose was tested in broths 1, 2, and 3, and maltose in broths 4, 5, and 6. Records of acid and gas production after incubation at 37° C. are given in Tables III and IV.

TABLE III

COMPARISON OF SACCHAROSE FERMENTATION AS INDICATED BY BROMTHYMOL BLUE AND BROMCRESOL PURPLE, USING STOVALL AND BUBOLZ'S, HOPKINS AND HESSELTINE'S, AND KUROTCHKIN'S BROTHS

| CULTURE NO. | FOURTH DAY | | | | | | TWELFTH DAY | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B. T. B. | | | B. C. P. | | | B. T. B. | | | B. C. P. | | |
| | S | H | K | S | H | K | S | H | K | S | H | K |
| | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID |
| 1 | 2 | 1 | 3 | 1 | – | 1 | 4 | 4 | 3 | 3 | 3 | 2 |
| 2 | 2 | 2 | 2 | 1 | 1 | 1 | 4 | 4 | 3 | 3 | 3 | 3 |
| 3a | 2 | 1 | 2 | 1 | 1 | 1 | 4 | 3 | 4 | 2 | 3 | 3 |
| 3b | – | – | 1 | – | – | 1 | 3 | 2 | 3 | – | – | 3 |
| 4 | 3 | 1 | 4 | 2 | 1 | 3 | 4 | 4 | 4 | 4 | 3 | 3 |
| 6 | 2 | 1 | 3 | 1 | 1 | 1 | 3 | 4 | 2 | 3 | 3 | 2 |
| 7 | 2 | 1 | 2 | 1 | 1 | 1 | 3 | 4 | 2 | 2 | 2 | 2 |
| 8 | 2 | 3 | 3 | 1 | 2 | 2 | 3 | 4 | 4 | 3 | 2 | 3 |
| 9 | 2 | 1 | 3 | 1 | 1 | 1 | 2 | 4 | 3 | 2 | 2 | 2 |
| 14 | 2 | 1 | 2 | 1 | 1 | 1 | 3 | 4 | 3 | 2 | 2 | 2 |
| 15 | 4 | 4* | 3 | 4* | 4* | 4* | 4* | 4* | 4* | 4* | 4* | 4* |

B. T. B.—Bromthymol blue
B. C. P.—Bromcresol purple
S—Stovall and Bubolz
H—Hopkins and Hesseltine
K—Kurotchkin
1, 2, 3, 4—Representing increasing degrees of acidity
*—Gas

TABLE IV

COMPARISON OF MALTOSE FERMENTATION AS INDICATED BY BROMTHYMOL BLUE AND BROMCRESOL PURPLE USING ASHFORD'S, DUNHAM'S PEPTONE, AND YEAST EXTRACT BROTH

| CULTURE NO. | FOURTH DAY | | | | | | FOURTEENTH DAY | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B. T. B. | | | B. C. P. | | | B. T. B. | | | B. C. P. | | |
| | AS. | D | Y | AS. | D | Y | AS. | D | Y | AS. | D | Y |
| 1 | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG |
| 2 | AG* | AG | AG | AG | AG | AG* | AG | AG | AG | AG | AG | AG |
| 3a | – | – | A | – | – | – | A | A | A | – | – | A |
| 3b | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | AG* | A | AG | AG* | AG | AG* | AG | AG* | AG | AG* | AG | AG* |
| 6 | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG |
| 7 | AG | AG | AG* | AG* | AG | AG | AG | AG | AG* | AG | AG | AG |
| 8 | A | A | AG | AG* | AG* | A | AG* | A | AG* | AG | AG* | AG |
| 9 | AG | AG | AG* | AG | AG* | AG | AG | AG | AG | AG | AG* | AG |
| 14 | AG* | AG* | AG | A | AG | AG | AG | AG* | AG | A | AG | AG |
| 15 | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG | AG |

B. T. B.—Bromthymol blue
B. C. P.—Bromcresol purple
As.—Ashford's
D—Dunham's peptone (Benham)
Y—Yeast extract
A—Acid
G—Gas
*—One tube of duplicates produced gas

Few differences were noticed between the various basal media used in the fermentations of saccharose and maltose. Differences were mainly in the amount of acid produced in saccharose, bromthymol blue indicating smaller

amounts of acidity than bromcresol purple. Some discrepancies in gas production in duplicate tubes were noted in maltose broth. Since none of the six broths appeared preferable from the standpoint of fermentation, standard methods beef extract broth (used by Stovall) was selected as a base, and bromthymol blue as the indicator for the remainder of the work. It was believed heat sterilization might cause hydrolysis of carbohydrates and account for anomalous fermentation results, so eight of ten carbohydrates used for identification of cultures were sterilized by passage through a Seitz filter, the exceptions being inulin and dextrin.

The effects of inoculating fermentation tubes with cultures from malt and from nutrient agars were then tested. Martin and coworkers (1937) emphasized the necessity of using inoculums from second or third generations on sugar-free media. Saccharose broth was employed in this work, omitting the vaseline seal method of Martin. Results obtained with inoculum from either malt agar or the third subculture on nutrient sugar-free agar with eleven cultures previously used are shown in Table V. The consistent results in this limited series did not indicate superiority for sugar-free agar, and all further inoculations were made from malt agar cultures.

TABLE V

COMPARISON OF SACCHAROSE FERMENTATION PRODUCED BY INOCULUMS FROM MALT AGAR AND FROM NUTRIENT AGAR GROWTH, AS INDICATED BY BROMTHYMOL BLUE AND BROMCRESOL PURPLE

| CULTURE NO. | THIRD DAY | | | | SEVENTH DAY | | | | FOURTEENTH DAY | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B. T. B. | | B. C. P. | | B. T. B. | | B. C. P. | | B. T. B. | | B. C. P. | |
| | M | N | M | N | M | N | M | N | M | N | M | N |
| | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID | ACID |
| 1 | 1 | 1 | – | – | 2 | 3 | 1 | 2 | 4 | 4 | 2 | 3 |
| 2 | 1 | 2 | – | 1 | 2 | 3 | 1 | 2 | 4 | 4 | 2 | 3 |
| 3a | 1 | 1 | – | – | 2 | 2 | 1 | 1 | 3 | 3 | 1 | 2 |
| 3b | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | 3 | 3 | 1 | 1 | 3 | 3 | 1 | 2 | 4 | 4 | 2 | 3 |
| 6 | 2 | 1 | – | – | 2 | 2 | 1 | 1 | 4 | 4 | 2 | 3 |
| 7 | 1 | 1 | 1 | – | 2 | 2 | 2 | 1 | 4 | 4 | 3 | 3 |
| 8 | 1 | 3 | 1 | 2 | 3 | 3 | 3 | 3 | 4 | 4 | 2 | 2 |
| 9 | 2 | 1 | – | – | 2 | 2 | 1 | 1 | 4 | 4 | 3 | 2 |
| 14 | 1 | 1 | – | – | 2 | 3 | 1 | 3 | 4 | 4 | 3 | 2 |
| 15 | 3 | 3 | 1 | 1 | 3 | 2 | 2 | 2 | 4 | 4 | 3 | 4 |

B. T. B.—Bromthymol blue
B. C. P.—Bromcresol purple
M—Inoculum from malt agar
N—Inoculum from third subculture on nutrient agar
1, 2, 3, 4—Represent increasing degrees of acidity

Ten carbohydrates were employed in this study, although for routine identification, as shown by Martin, dextrose, saccharose, lactose, and maltose would suffice. The other six carbohydrates used were levulose, mannose, galactose, raffinose, inulin, and dextrin. Records of acid and gas production were made at the end of one and two weeks' incubation. The fermentation reactions with thirteen cultures from pathogenic conditions are shown in Table VI.

*Colony Morphology on Malt Agar.*—Preliminary separation of dissimilar strains of yeastlike fungi was made by selection of those having different

colony appearance after forty-eight hours of incubation on acidified malt agar. The size of surface and subsurface colonies, and amount and appearance of mycelial radiations with thirteen cultures from pathogenic conditions are given in Table VII. Subsurface colonies were generally smaller than those on the surface, produced mycelium more profusely and sooner. Few strains produced surface mycelium in forty-eight hours.

*Growth in Corn Meal Agar.*—Cultures were inoculated by making deep streaks in Difco medium, and growth was observed in situ, using low power of the microscope at intervals during two weeks' incubation at room tempera-

TABLE VI

FERMENTATION REACTIONS OF THE YEASTLIKE FUNGI ON TEN CARBOHYDRATES

| CULTURE NO. | DEXTROSE | LEVULOSE | MANNOSE | SACCHAROSE | MALTOSE | GALACTOSE | LACTOSE | RAFFINOSE | INULIN | DEXTRIN |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | AG | AG | AG | A | AG | A | – | – | – | A |
| 2 | AG | AG | AG | A | AG | A | – | – | – | A |
| 3a | A | A | AG | A | A | AG | – | – | – | – |
| 3b | A | A | A | – | – | A | – | – | – | – |
| 4 | AG | AG | AG | A | AG | A | – | – | – | A |
| 6 | AG | AG | AG | A | AG | A | – | – | – | A |
| 7 | AG | AG* | AG | A | AG | A | – | – | – | A |
| 8 | AG | AG | AG | A | AG | A | – | – | – | A |
| 9 | AG | AG | AG | A | AG | A | – | – | – | A |
| 14 | AG | AG | AG | A | A | A | – | – | – | A |
| 15 | AG | AG | AG | A | AG* | A | – | – | – | A |
| 30a | A | A | A | – | – | A | – | – | – | – |
| 30b | A | A | A | A | A | A | A | A | – | A |

*—One tube of duplicates produced gas
A—Acid
G—Gas

ture. Some parts of growth were also examined through the high dry lens, after placing a cover slip on the agar surface. These observations allowed differentiation of gross mycelial structures, and placement of the organisms into tentative groups. Strains identified as *Monilia albicans* produced both branched and beaded growths, necessitating a thorough search in order to find chlamydospores, which were not observed in all cultures. Mycelial descriptions are presented in Table VII.

*Detection of Ascospores.*—Carrot slants, sterilized in tubes containing portions of cotton in the bottom which were moistened from time to time, were inoculated and kept for two and one-half months at room temperature. The preparations were examined for asci at intervals. Mounts in plain water were satisfactory for showing ascospores. A double staining technique for ascospores using carbolfuchsin and methylene blue was also effective.

*Growth in 1 per cent Dextrose Broth.*—Each culture was inoculated into 1 per cent dextrose broth, pH 6.0, and was incubated at 37° C. for forty-eight hours. The type of growth was noted, whether pellicle, ring, or flakes adher-

TABLE VII

COLONY APPEARANCE OF YEASTLIKE FUNGI ON MALT AGAR AND MYCELIUM PRODUCTION IN CORN MEAL AGAR

| CULTURE NO. | MALT AGAR COLONY, 48 HOUR | | | | CORN MEAL AGAR |
|---|---|---|---|---|---|
| | SURFACE | | SUBSURFACE | | BRIEF DESCRIPTION OF MYCELIUM |
| | MYCELIUM | SIZE MM. | MYCELIUM | SIZE MM. | |
| 1 | 0 | 2.0 | 0 | 0.5 | Scanty, much branched, typical of *M. albicans*, chlamydospores |
| 2 | 0 | 2.0 | 0 | 0.75 | Scanty, much branched, typical of *M. albicans*, chlamydospores |
| 3a | 0 | 1.5 | + | p. pt. | Medium amount, parallel bushy rows from streak |
| 3b* | 0 | 0.75 | 0 | p. pt. | No mycelium |
| 4 | 0 | 2.5 | 0 | 0.75 | Scanty, much branched and beaded, typical of *M. albicans* |
| 6 | 0 | 2.5 | 0 | 0.75 | Scanty, branched and beaded, typical of *M. albicans* |
| 7 | 0 | 2.5 | 0 | 0.75 | Scanty, branched and beaded, typical of *M. albicans* |
| 8 | 0 | 1.75 | + | 1.0 | Scanty, much branched, suggested *M. albicans* |
| 9 | 0 | 2.5 | 0 | 0.75 | Scanty, branched and beaded, like *M. albicans*, chlamydospores |
| 14 | 0 | 2.0 | 0 | 0.75 | Scanty, branched and beaded, like *M. albicans*, chlamydospores |
| 15 | 0 | 2.0 | 0 | 0.75 | Scanty, branched and beaded, typical of *M. albicans* |
| 30a | + + | 1.25 | + + + | p. pt. | Long, rarely septate, bunches of conidia from short branches |
| 30b | 0 | 0.75 | + | p. pt. | Long, arthrospores, branched, fat globules, chlamydospores |

*Four days' growth
0 No mycelium
+, + +, + + + Increasing length
p. pt. Pinpoint

TABLE VIII

CELLULAR CHARACTERISTICS OF YEASTLIKE FUNGI AND TYPE OF GROWTH IN 1 PER CENT DEXTROSE BROTH AFTER 48 HOURS

| CULTURE NO. | CELL SIZE μ | GROUPING* | INTERNAL STRUCTURE | DEXTROSE BROTH |
|---|---|---|---|---|
| 1 | 3.75 | Small clumps | Small vacuole | SFR |
| 2 | 3.75 | Small clumps | Small vacuole | SFR |
| 3a | 3.75 | Small clumps | Medium vacuole | S |
| 3b | 4.05 | Small clumps and pairs | Large vacuole | SF |
| 4 | 4.05 | Medium clumps | Small vacuole | SFR |
| 6 | 4.05 | Small clumps | Small vacuole | SFR |
| 7 | 3.45 | Large clumps | Small vacuole | SFR |
| 8 | 3.9 | Large clumps | Small vacuole | SFR |
| 9 | 3.45 | Large clumps | Small vacuole | SFR |
| 14 | 3.3 | Small clumps | Small vacuole | SFR |
| 15 | 3.6 | Small clumps | Small vacuole | SFR |
| 30a | 7.0 × 2.25 | Singly, pairs, mycelium | 1-2 vacuoles | STR |
| 30b | 6.3 × 4.05 | Branched chains, mycelium | Large vacuole | SF |

*Small clumps averaged less than 6 cells; medium 6; large more than 6
S—Sediment
F—Flakes adhering to sides of tube
R—Ring
T—Turbidity

ing to the sides of the tube, turbidity or sediment at the bottom. Hanging drops were examined for cell grouping and stained smears were also examined for cell size and internal structure. These results are given in Table VIII.

*Coagulation of Milk.*—Milk prepared according to directions of Stovall and Bubolz (1932) was inoculated from forty-eight-hour cultures by means of a

loop needle, and incubated at 37° C. for four weeks, examining daily for the first week and weekly thereafter for coagulation. Duplicate tubes were not inoculated, therefore coagulation of milk was not given major importance in classification, even though stained smears were made from tubes showing coagulation to rule out possible contamination by bacteria. The time of milk coagulation is given in Table IX.

TABLE IX

COAGULATION OF MILK, GROWTH IN GELATIN FOR ONE MONTH AT 20° C. AND TYPE OF GROWTH BY YEASTLIKE FUNGI ON MALT AGAR SLANTS AFTER 48 HOURS

| CULTURE NO. | TIME OF MILK COAGULATION DAYS | GROWTH IN GELATIN | GROWTH MALT AGAR SLANTS |
|---|---|---|---|
| 1 | 2 | Nail | Smooth, white, shiny |
| 2 | 2 | Nail | Smooth, white, shiny |
| 3a | - | Nail | Smooth, white, shiny |
| 3b | 2 | Nail | Smooth, pink, shiny |
| 4 | 3 | Nail | Smooth, white, shiny |
| 6 | 3 | Nail | Smooth, white, shiny |
| 7 | 2 | Nail | Smooth, white, shiny |
| 8 | 2 | Nail | Smooth, white, shiny |
| 9 | 2 | Nail | Smooth, white, shiny |
| 14 | 2 | Nail | Smooth, white, shiny |
| 15 | 3 | Nail | Smooth, white, shiny |
| 30a | - | Pine tree | Rough, white, dull |
| 30b | 3 | Liquefaction | Corrugated, white, mucoid |

*Growth in Gelatin.*—Ten per cent beef extract gelatin was prepared, adjusting the reaction to pH 7.2 before autoclaving. Tubes of medium were stabbed, using forty-eight-hour growth, and incubated at 20° C. for one month. Appearance in gelatin differed slightly from that reported by other investigators in that mycelial radiations were absent in most cultures, due perhaps to the incubation temperature used. The type of growth obtained is recorded in Table IX.

*Growth on Malt Agar Slants.*—The type of growth obtained on malt agar slants after forty-eight hours' incubation at 37° C. is also recorded in Table IX.

*Classification.*—Nine of the thirteen strains from pathogenic conditions were classified as *Monilia albicans.* Of the remaining four, one was an unidentified Monilia species (3a), one a pink cryptococcus (3b), and two were unidentified yeastlike fungi (30a, 30b).

## DISCUSSION

Comparison of plating media, enrichment broth pH and incubation times and temperatures, and basal media for fermentation reactions, have already been discussed in detail in their respective sections of this report. Our results show that simple plating and fermentation media are satisfactory in studying yeasts and yeastlike fungi. However, care must be given to such factors as suitable pH, avoidance of hydrolysis of carbohydrates during sterilization and proper inoculation.

Variable fermentation reactions, chiefly failure of gas production in duplicate tubes, were observed, even though particular care was taken to eliminate variations in media and method of inoculation. Observations suggest that

compact yeast growth may block the entrance to inverted gas tubes, thus deflecting all gas produced in quantities too large to be absorbed in the medium. For this reason, the vaseline seal method of Martin and associates may prove of value, since these workers reported consistent reactions. Fisher and Arnold stated that biochemical reactions should be repeated to determine the most accurate results. The nine cultures of *Monilia albicans* gave the most consistent fermentation results of any cultures studied, producing acid and gas in dextrose, levulose, mannose, and maltose, and acid in saccharose, galactose, and dextrin.

The degree of mycelium production and size of surface and subsurface malt agar colonies was not a consistent differential characteristic, variations within a Monilia species being common. A thorough examination of corn meal agar growth greatly aided identification, although growth morphology was not constant, e.g., chlamydospores were not regularly observed in *Monilia albicans*. Martin and others believed inoculation of corn meal agar from nutrient agar alleviated some inconsistency. Determinations for ascospores might be made from old growth on malt agar slants, or corn meal agar plates, to verify or hurry observations on carrots, the latter also recommended by Martin and coworkers, and found desirable in the present study. However, with cultures isolated in this study the use of blood agar plates as recommended by Martin and associates did not yield added information.

Biochemical and morphologic methods of classification were found to be sufficient for identification of most of the yeastlike fungi isolated, our work agreeing in this respect with that of Fisher and Arnold. Accordingly it did not seem necessary to resort to serologic methods or to the criteria presented by growth of giant colonies, inasmuch as these are time consuming and may likewise be subject to variation. Maltose and saccharose fermentations (employed by Stovall) yielded the most important differential characteristics, however, it was felt that dextrose and lactose (used by Martin) gave sufficient additional information to be of value. The remainder of the ten carbohydrates were not necessary for routine identification.

The system of classification outlined by Fisher and Arnold (1936), following Castellani, was followed as closely as possible in this work. This is believed to be a readily interpreted key, since emphasis has not been placed on minute morphologic details.

## SUMMARY

1. Malt agar gave consistently high counts, and well-defined colonies of yeastlike fungi in comparisons with honey agar or Sabouraud's maltose or dextrose agars.

2. Enrichment studies in honey broth, pH 4.1 to 5.8, incubated at varying times and temperatures indicated that pH 5.4 to 5.8 for twenty-four hours at room temperature was most favorable for increased numbers of yeast cells. However, broth at pH 5.0 to 5.4 was less advantageous for bacterial multiplication.

3. Standard methods beef extract broth as a base for fermentation media seemed to give as satisfactory results as any of five special basal media that have been used for this purpose.

4. Results obtained in inoculating a limited series of fermentation tubes with cultures from malt or nutrient agars did not indicate superiority of inoculums from sugar-free agar, thus eliminating the necessity for subculturing on the latter medium for several generations.

5. Preliminary separation of yeast strains was made by selection of those having different colony appearance after forty-eight hours of incubation on acidified malt agar. Subsurface colonies were generally smaller than those on the surface, and produced mycelium more profusely and sooner. Few strains produced surface mycelium in forty-eight hours, and this was not found to be a consistent differential characteristic.

6. Inoculation of cultures into corn meal agar by deep streaks, and observations of resulting growth in situ during two weeks' incubation at room temperature, allowed placement of organisms into tentative groups. Giant colonies were not considered for use because of time required and because of variability reported.

7. Although observation of cellular morphology from dextrose broth was valuable in some instances, the main value of this medium in other studies was the differentiation possible when pellicle formation occurred. Type of growth in gelatin did not give much aid in the classification of yeastlike fungi, whereas the coagulation of milk seemed valuable as a differential characteristic, but not on the basis of a single test.

8. Fermentation reactions in four carbohydrates, namely, dextrose, saccharose, maltose, and lactose, proved valuable in separation of species, but absolute reliance was not placed on these reactions because of variations which occurred.

9. Morphologic observations of colonies on malt agar, growth in corn meal agar, detection of ascospores from growth on carrot slants, and cultural features, such as growth in 1 per cent dextrose broth, coagulation of milk, growth in gelatin, type of growth on forty-eight-hour malt agar slants, and fermentation reactions in at least four carbohydrates, enabled separation of yeastlike fungi into genera, and into species where these had been reported.

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# YEASTLIKE FUNGI ISOLATED FROM NORMAL SKINS*

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NUMEROUS associations of yeasts and yeastlike fungi with pathologic skin conditions have been reported. These organisms have also been found occurring on healthy skin, and their pathogenicity has been questioned. This study of yeastlike fungi from a selected area of normal skin, the finger tip, was undertaken to ascertain the presence and frequency of such organisms, and to compare fungi found with those isolated by other workers.

Greenbaum and Klauder (1922) examined the skin of the axillae, inguinal folds, and interdigital spaces of toes and fingers of 150 normal patients. They classified 35 strains isolated into four types of yeast, three as Saccharomyces, and one as Cryptococcus. Jessner and Kleiner (1925) studied the finger nails of 150 persons and found budding fungi from 58 per cent of men and 60 per cent of women. When transferred to macerated and scarified skins of normal interdigital spaces, certain yeast strains isolated, which killed mice, produced infection in forty-eight hours.

Falchi (1926) cultured fungi from 37 of 57 normal skins. Yeasts were found four times, including Saccharomyces twice, and Cryptococcus twice. Cornbleet (1926) obtained a variety of common molds and yeast from apparently normal feet. Wachowiak and others (1929) failed to cultivate Monilia or similar organisms from skin scrapings of the elbow region of 26 normal persons. Staehelin and associates (1932) cultured skin of various body surfaces of 120 persons, 64 of whom were normal and 56 suffering from dermatoses. Yeasts were isolated from normal skins in more instances (82.8 per cent) than from diseased skins (71.4 per cent). Fourteen of the strains isolated were believed to be Monilia.

More recently, Benham and Hopkins (1933) classified yeastlike fungi isolated from skin, nails of fingers, web between fourth and fifth toes, and nail of the great toe, of 72 of the 100 people examined. From the fingers they obtained one strain of *Monilia parapsilosis*, one of *Monilia krusei*, two unidentified Monilia, 49 Cryptococci, and two Mycodermata. *Monilia albicans* was not isolated from the normal skin.

Fisher and Arnold (1936) cultured the skin and mucous membranes of 76 persons. Seventy-five per cent of adults and 52 per cent of children were found to have one or more strains of organisms which were classified as Sac-

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charomyces, Cryptococcus, Monilia, and Mycoderma. Endomycetes were not found, nor was *Monilia albicans* cultured from normal skin, although it was recovered from mucous membranes, gastric contents, and feces from healthy people. Todd (1937) found *Monilia albicans* in the mouths and throats of 14 per cent of 1,000 normal individuals.

Downing and collaborators (1937) isolated 22 filamentous fungi and 16 yeastlike fungi from behind the ears, corners of the mouth, and the toes of 100 people.

## METHODS

The tip of the middle finger on the right or left hand was selected as an accessible site for isolating yeastlike fungi. Any organisms being present, even though recently acquired from an extraneous source, would be considered potentially infective, and could be transferred to or from any part of the body; therefore, the subjects were not asked to wash their hands.

In sampling, a finger of each of the 100 persons was first soaked for three to five minutes in 5 c.c. of sterile honey broth contained in a small snap-cap jar. A thorough scraping was then given the finger tip, using an autoclaved scalpel blade, held in flamed hemostatic forceps. Care was taken to scrape bits of skin, nail, and substance under the nail directly into the honey broth, a method similar to that used by Benham and Hopkins (1933). From the first 25 people sampled, a 2 c.c. portion of the honey broth was then plated during the first hour after scraping, using Difco malt extract agar. The agar was acidified to approximately pH 4.0 before plating by the addition of 1 c.c. of a $\frac{1}{10}$ dilution of lactic acid, C.P., to 100 c.c. of melted agar, a degree of acidity that prevented growth of bacteria. The remaining 75 finger scrapings in honey broth of pH 5.0 to 5.4 were incubated at room temperature for twenty-four hours before plating, previous enrichment experiments (Croft and Black, 1938) having shown this was most favorable for multiplication of yeastlike fungi. The malt agar plates were incubated at 37° C. for one week before discarding.

Cultures, when isolated, were replated from sufficiently high dilutions in sterilized tap water to produce well-isolated colonies on acidified malt agar. These were fished to malt agar slants, pH 5.0, and the pure cultures were kept at room temperature, being transferred at intervals of four weeks, or oftener, to fresh malt agar slants.

The yeastlike fungi isolated were classified on the basis of the following reactions which had been found suitable in our previous studies:

1. Forty-eight-hour colony mycelial radiations on malt agar.

2. Forty-eight-hour to two-week-old mycelial formation in corn meal agar.

3. Detection of ascospores from one and one-half to two and one-half-month-old growth on carrot plugs by examining aqueous suspensions and stained films.

4. Fermentation reactions in beef extract broth containing either dextrose, levulose, mannose, saccharose, maltose, galactose, lactose, raffinose, inulin, or dextrin in 1 per cent concentration, and bromthymol blue indicator. The

carbohydrates were sterilized by filtration, with the exceptions of inulin and dextrin, and added to the broth aseptically. The tubes were inoculated with a small amount of growth from a forty-eight-hour malt agar slant culture, and records were made at the end of one and two weeks' incubation at 37° C.

5. Type of forty-eight-hour growth in 1 per cent dextrose broth.

6. Coagulation of milk prepared according to the method of Stovall and Bubolz (1932).

7. Growth in 10 per cent beef extract gelatin during one month's incubation at 20° C.

## RESULTS

Yeast and yeastlike fungi were isolated from the finger tips of 22 of 100 persons examined. The incidence of positive isolations among various groups of individuals is shown in Table I. Persons whose hands were kept moist much of the time showed a greater percentage of yeasts, e.g., kitchen workers of a large institution, 87.5 per cent; laboratory apparatus washers, 75 per cent; and housewives, 33.3 per cent. In four instances two different yeastlike fungi were isolated from a single finger and from one individual four strains were obtained. The incidence for women was slightly higher than for men.

TABLE I

ISOLATIONS OF YEASTLIKE FUNGI UPON SINGLE EXAMINATION OF A MIDDLE FINGER OF NORMAL INDIVIDUALS

| | NUMBER SAMPLED | NUMBER POSITIVE | PER CENT POSITIVE |
|---|---|---|---|
| University students, or professors (Total) | 59 | 7 | 11.9 |
| University women | 14 | 1 | 7.1 |
| University men | 45 | 6 | 13.3 |
| Laboratory apparatus washers | 4 | 3 | 75.0 |
| Housewives | 9 | 3 | 33.3 |
| Kitchen workers | 8 | 7 | 87.5 |
| Library workers | 8 | 2 | 25.0 |
| Miscellaneous | 15 | 2 | 13.3 |
| Total men | 68 | 14 | 20.6 |
| Total women | 32 | 8 | 25.0 |
| Total | 100 | 22 | 22.0 |

Twelve of 29 cultures of yeast and yeastlike fungi isolated from normal skins were classified as being similar or closely related to *Monilia parapsilosis*. Previous workers have reported different fermentation characteristics for this species, particularly the fermentation of maltose and saccharose. Stovall and Bubolz (1932) reported the production of acid in both sugars, Benham (1931) found acid only in saccharose, whereas Martin and coworkers (1937) and also Fisher and Arnold (1936) reported the absence of fermentation with these carbohydrates. Variations in carbohydrate fermentation were noted, but the mycelial development of the strains was similar and characteristic. Variation in milk coagulation was also obtained with six of the strains coagulating milk. These results on milk coagulation apparently are confirmed in a recent study by Worley and Stovall (1937) who stated: "In the case of *M. parapsilosis*, contrary to our previous reports in which we obtained no coagulation of calcium lactate milk, it appears that there are certain cultures which do produce a coagulating agent, and others which do not."

TABLE II

CHARACTERISTICS OF MONILIA ISOLATED FROM NORMAL SKINS

| | | CULTURE NO. | CARBOHYDRATE REACTIONS | | | | | | | | | | CULTURAL FEATURES | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | GROWTH | | MYCELIAL LENGTH | | |
| | | | | | | | | | | | | | | | | MALT AGAR COLONIES | | |
| | | | DEXTROSE | LEVULOSE | MANNOSE | SACCHAROSE | MALTOSE | GALACTOSE | LACTOSE | RAFFINOSE | INULIN | DEXTRIN | MILK COAG. DAYS | GELATIN | DEXTROSE BROTH | SURFACE | DEEP | CORN MEAL AGAR STREAK |
| *Monilia parapsilosis* (Or closely related) | St | 16 | AG | AG | AG | A | A | A | – | – | – | – | – | N | SF | – | + | + |
| | | 20 | AG | AG | AG | A | A | A | – | – | – | – | – | N | SFR | – | – | + |
| | St? | 28 | AG | AG | AG | A | A | AG | – | – | – | – | – | N | SFR | – | ++ | + |
| | | 33a | AG | AG | A | A | A | AG | – | – | – | – | – | N | SFR | Ro | + | + |
| | | 34 | AG | AG | AG | A | A | AG | – | – | – | – | 6 | N | SFR | – | – | + |
| | | 26 | AG | AG | AG | A | A | A | – | – | – | – | 5 | N | SFR | – | + | + |
| | | 23c | AG | AG | A | A | A | A | – | – | – | – | 6 | N | SFR | – | ++ | + |
| | | 31 | AG | AG | A | A | A | A | – | – | – | – | 4 | N | SF | – | + | + |
| | | 35a | AG | AG | A | A | A | A | – | – | – | – | 4 | N | SFR | – | – | + |
| | | 36a | AG | A | AG | A | A | A | – | – | – | – | 6 | N | SFR | – | + | + |
| | B | 18a | AG | AG | AG | A | – | A | – | – | – | – | – | N | SFR | – | + | + |
| | M, F-A | 24 | AG | AG | AG | – | – | A | – | – | – | – | – | N | SFR | – | Ro | + |
| *Monilia nigra* | | 22 | A | A | A | A | A | A | – | A | – | A | – | L | F | +++ | +++ | +++ |
| | | 36b | A | A | A | A | A | – | – | – | – | A | 2 | L | SFR | Ro | + | +++ |
| | | 38 | A | A | A | A | A | – | – | – | – | A | 3 | L | SFR | Ro | + | +++ |
| Unidentified Monilia species | | 18b | AG | AG | AG | A | A | A | – | – | – | – | – | N | SFP | – | + | ++ |
| | | 23a | AG | AG | A | – | A | A | – | – | – | – | 14 | N | SFR | – | – | ++ |
| | | 33b | AG | AG | AG | A | A | A | – | A | A | – | 28 | N | SF | – | – | ++ |

St—Stovall
B—Benham
M—Martin
F-A—Fisher and Arnold

A—acid
G—gas
N—nail
L—liquefied

S—sediment
F—flakes
R—ring
P—pellicle
Ro—rough edge

TABLE III

CHARACTERISTICS OF YEASTS AND YEASTLIKE FUNGI OTHER THAN MONILIA ISOLATED FROM NORMAL SKINS

| YEASTS AND YEASTLIKE FUNGI | CULTURE NO. | CARBOHYDRATE REACTIONS | | | | | | | | | | CULTURAL FEATURES | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | GROWTH | | MYCELIAL LENGTH | | |
| | | | | | | | | | | | | | | | MALT AGAR COLONIES | | |
| | | DEXTROSE | LEVULOSE | MANNOSE | SACCHAROSE | MALTOSE | GALACTOSE | LACTOSE | RAFFINOSE | INULIN | DEXTRIN | MILK COAG. DAYS | GELATIN | DEXTROSE BROTH | SURFACE | DEEP | CORN MEAL AGAR STREAK |
| Endomyces species | 19 | A | A | AG | - | - | - | - | - | - | - | - | N | SFP | Ro | + | + |
| | 21 | AG | AG | AG | AG | A | AG | - | A | A | A | - | N | SF | - | - | + |
| | 27 | AG | AG | AG | AG | - | AG | - | A | A | - | - | N | SFR | - | +++ | ++ |
| | 29 | AG | AG | AG | AG | - | AG | - | A | A | - | 28 | N | SR | - | +++ | ++ |
| Cryptococcus species | 37 | A | A | A | A | - | - | - | | - | - | 2 | N | SR | - | - | - |
| | 35b | AG | AG | AG | AG | AG | AG | - | A | - | - | - | N | SF | - | - | - |
| Mycoderma species | 25 | A | A | A | - | A | A | A | - | - | A | 5 | L | SFP | Ro | ++ | +++ |
| *Schizosaccharomyces hominis* | 17 | A | A | A | A | A | A | A | A | A | A | 3 | L | SFRP | - | - | - |
| Unidentified Yeastlike fungi | 23b | A | A | A | A | A | - | - | - | - | A | - | FU | SFR | | ++ | +++ |
| | 23d | A | - | AG | A | A | A | - | - | - | - | 14 | L | SFRP | +++ | +++ | +++ |
| | 32 | AG | AG | AG | A | A | A | - | - | - | A | - | PT | SFR | - | ++ | +++ |

A—acid
G—gas
N—nail
L—liquefied
FU—fuzzy
PT—pine tree
Ro—rough edge
S—sediment
F—flakes
R—ring
P—pellicle

The other generally recognized species of Monilia, *Monilia candida*, *Monilia krusei*, and the pathogen *Monilia albicans* were not recovered from the normal skins examined.

Three yeastlike fungi producing black mycelium were isolated. They were apparently similar to the group described by Henrici (1930) and by Guillermond and Tanner (1920), known as *Monilia nigra*, isolated from raw sugar and dairy products. When first isolated, the colonies were colorless and mucoid, but with age they became black, tough, and membranous. At this stage, black, thick-walled arthrospores were formed in addition to budding conidia from the mycelium.

Three strains were identified as Monilia species, but they did not correspond with any known type.

Four of the cultures of yeastlike fungi isolated were classified as Endomyces species. Two of these produced true ascospores, strain 19 usually four in an ascus, and strain 21 producing two in an ascus. The mycelium of these strains was scanty, much branched, and made up of short yeastlike cells. Strains 27 and 29 produced many kidney-shaped free spores, but spore sacs were not observed.

Cryptococcus cultures isolated were two in number, one of which was pink, corresponding to Benham's (1935) group IV. The other, strain 35b, corresponded to Benham's group I.

One Mycoderma was isolated. This strain produced a dry, wrinkled cream-colored growth on malt agar, and a pellicle on liquid media.

Culture 17 agreed with the description of *Schizosaccharomyces hominis* described by Benedek in 1927 (as reported by Gay [1935]). This organism has been isolated from types of seborrheic dermatitis. Other investigators doubted its pathogenicity, and considered it a harmless parasite. The cells resembled bacilli, and each produced a single spore. The grouping from a broth culture was in short chains, division occurring by fission. Pairs and single cells, some of which were rounded on one end and pointed on the other, were seen from agar cultures, and the cultures gave a yeastlike odor.

Yeastlike fungi which were not identified were three in number. They produced a long mycelium, nonseptate, or septate at rare intervals, with few conidia. Culture 23b produced a gray-black pigment.

The groupings and cultural reactions of these yeastlike fungi are presented in Tables II and III.

## DISCUSSION

The isolation of yeastlike fungi from 22 per cent of the individuals tested is somewhat lower than found by four other investigators. This is perhaps due to the fact that a selected area of the skin was cultured, whereas others sampled from more varied locations, with the exception of Jessner and Kleiner, who studied finger nail flora. In the present investigation, 59 of the subjects whose fingers were sampled were bacteriologic workers whose hands might be exposed to the action of disinfectants oftener than the average person's. This fact should be considered in interpreting the lower percentage of positive isolations.

The evidence that individuals whose hands were exposed to moisture a large part of the time had more yeastlike fungi on their fingers, was not in accord with that of Jessner and Kleiner. They concluded that occupation of the individual did not affect incidence to the presence of yeasts.

The yeasts isolated were not necessarily normal inhabitants of the skin and nails. Some of the cultures may have been transferred to the fingers from extraneous sources before sampling.

*Monilia albicans* was not obtained, nor were known pathogens found. However pathogenicity studies were not carried out on any of the strains isolated.

The number of Monilia isolated was higher than that found by Benham and Hopkins, or Fisher and Arnold. Sixteen organisms were identified as Monilia in this work, while Benham and Hopkins found four from 100 fingers, and Fisher and Arnold found 3 from 28 fingers. Benham and Hopkins' 58 Cryptococci outnumbered the two of that genus found in this study. The four Endomycetes we isolated are the only ones that have been reported from normal skin. It is possible that black filamentous forms found by Jessner and Kleiner were the same as *Monilia nigra* found in this study.

Since the twenty-four-hour enrichment isolation method was not carried out on the first 25 finger scrapings, the number of isolations of yeastlike fungi from that group might have been slightly lower than had enrichment methods been employed.

It seems safe to conclude, from evidence presented in this work, and from the work of previous investigators, that yeastlike fungi can live on the normal skin saprophytically.

## SUMMARY

Scrapings from the finger tips and nails of 100 people yielded 29 cultures of yeastlike fungi from 22 of the individuals sampled.

Persons whose hands were kept moist a large part of the time (kitchen workers, laboratory apparatus washers, and housewives) showed a higher incidence to yeastlike fungi than those with drier hands.

Twelve cultures were classified as being similar or closely related to *Monilia parapsilosis*, three as *Monilia nigra*, and three unidentified Monilia species.

Four Endomyces species were isolated. These are the first organisms of this type known to be reported from normal skin. Two Cryptococcus species, one Mycoderma species, one *Schizosaccharomyces hominis*, and three yeastlike fungi that could not be identified were also isolated.

*Monilia albicans*, or any known pathogenic yeastlike fungus, was not isolated from the normal skins.

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# THE GLUTATHIONE CONTENT OF BLOOD DURING THE PUERPERIUM*

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OF ALL the substances of the body having physiologic functions there are perhaps few, if any, which have more interesting properties than glutathione. Its manifold and diversified characteristics cause it to play a role as a protector of ascorbic acid (Hopkins); as an activator or coenzyme of glyoxalase (Lohmann); as reducing agent in its reduced form and as an oxidizing agent or more specifically, as a hydrogen acceptor in its oxidized state.

Glutathione is distributed throughout the body in the organs and muscles. Cerbone, however, was not able to demonstrate its presence in amniotic fluid. In blood, glutathione is confined largely, if not entirely, to the corpuscles, and it was to emphasize this that Gabbe proposed the expression $\frac{\text{GHS}}{\text{R.B.C.}}$ in which GHS represents milligrams of glutathione per 100 c.c. blood and R.B.C., millions of red blood cells per cubic millimeter of blood.

Though Woodward and Fry confirmed Gabbe's statement regarding the ratio of glutathione and red blood cells, they believe that the quotient should be

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modified so that it indicates approximately the oxidizing power of the red blood cells per unit number of cells. They, therefore, proposed the ratio $\frac{GHS \times Hb.}{R.B.C.}$ wherein they have included hemoglobin expressed as a percentage of normal. They find that the constancy of this value suggests that there may be a compensatory relationship between the amount of hemoglobin and the glutathione in the blood, or that the hemoglobin may determine that fraction of glutathione which occurs in the reduced form in venous blood.

Pickard and Marsden do not concur with these findings, believing that their results show that the ratios of Gabbe and Woodward and Fry are not indicative of any relationship between these elements in normal blood. Oberst and Woods have also failed to find a correlation between total glutathione and oxygen capacity or hemoglobin and between percentage of reduced glutathione and percentage of oxygen saturation, although they do state that in general higher total glutathione values in whole blood are generally associated with higher cell volumes and oxygen capacity.

Bach and Bach in a study of catalase and glutathione content of the red blood cells in anemia found that the amount of each substance is increased in the individual red cell in pernicious anemia. Kumagai obtained the same results in anemia produced in rabbits by acute bleeding or by acute poisoning with phenylhydrazine. He found that the catalase and glutathione content of the blood rose more rapidly than the erythrocyte count or hemoglobin content, the oxygen capacity of the blood being increased in relation with the increase in catalase and glutathione content of the blood.

Anselmino and Hoffmann have observed that the glutathione and catalase content of fetal blood is higher than that of maternal blood. Their results show that fetal blood contains about 40 per cent more glutathione than maternal blood. Cerbone, Schüler, Buzzi, Sala, Lemeland, and Deletang and others agree that fetal blood contains a higher concentration of glutathione than maternal blood. Lemeland and Deletang believe the placenta to be permeable to glutathione, since they found the same values for the glutathione content of the uterus, placenta, and fetus. Dogliotti states that the high glutathione content of fetal blood may be accounted for by the greater concentration of red corpuscles. Furthermore, he found the placenta to be a depot for the storage of glutathione, but is of the opinion that it in itself does not enter the oxidation-reduction system.

A survey of the evidence then shows that there may be no direct relationship between glutathione and cell volume, red blood cell count, oxygen capacity, or hemoglobin. It may be that glutathione exerts its maximum oxidation-reduction properties in conjunction with catalase. However, one is left with the impression that following hemorrhage, or in diseases in which there is a stimulation of the bone marrow, the red corpuscles have a high content of glutathione.

Stander and Cadden have reported that the glutathione content of blood during eclampsia is within normal limits. They have observed a decrease during the early puerperium and have associated this decline with low hemoglobin values resulting from the bloss of blood at delivery. In view of the recent studies on the changes of cell volume during labor and puerperium, particularly

in patients with pre-eclampsia or eclampsia, it is possible that the changes which they observed may be due to changes in cell volume or hemoglobin which are not associated with blood loss. Skajaa and Dieckmann have reported marked concentration of the blood during eclampsia. Skajaa states that an average blood concentration of 15 per cent occurs during labor in toxemic patients. Dieckmann does not believe that the concentration of the blood is the cause of eclampsia, but both he and Skajaa believe that a subsequent dilution of the blood is associated with clinical improvement.

It might be pointed out here that in the interpretation of cell volume variations one should not presume that they necessarily represent alterations of the water content of blood. An increase in cell volume may represent one of several changes. For example, there may be an absolute increase in the number of cells in the vascular system while the absolute plasma volume may remain the same; there may be an absolute decrease in the plasma volume while there is either no change or an increase in the number of cells in the system. The decrease in plasma volume may, in turn, be altered in two ways. It may be concentrated by the loss of water or its volume may be decreased by a removal of part of the plasma from the vascular system through the loss of water and the soluble elements of the plasma. Still another change which may be reflected in a change of cell volume is that the individual cells themselves may become enlarged. Thus one should be cautious in either asserting or interpreting that cell volume changes indicate a concentration or dilution of the blood. The term "concentration of blood" should be applied when one means that water is removed or added to the system and not when the change is a physical one.

Since glutathione is involved in the oxidation-reduction phenomenon of the body and perhaps in carbohydrate metabolism, as evidenced by its action on glyoxalase, and has within its molecule the amino acids glycine and glutamic acid which may be considered detoxicating agents, it is a substance which can be of interest to those who believe that eclampsia is caused by some toxic substance as well as to those who are of the opinion that an alteration in the oxidation system plays an important role in eclampsia. In regard to the latter view, it has been briefly stated by Stander that "any theory which is to explain the etiology of eclampsia will have to be linked up in some way or other with deficient oxidation."

## EXPERIMENTAL

A study has been made of the glutathione content of the blood of 40 women during labor and the puerperium. In order to reduce to a minimum the number of venepunctures performed on each case, the patients were divided into two groups of 20 cases each. In the first group blood was drawn on the first, third, fifth, seventh, and ninth days of the puerperium. In the other group blood was drawn on the second, fourth, sixth, eighth, and tenth days post partum. While each group represents 20 cases, I believe that a composite graph obtained from the averages of both groups should represent the variations for the entire series.

The determination of glutathione was carried out on the blood by the procedure of Benedict and Gottschall on a 1:10 tungstomolybdic filtrate. The stock solution of glutathione was made every four weeks and from this a working standard was prepared at least twice a week.

In Tables I and II are given the values obtained from the individual cases. A study of the averages shows that the glutathione of the blood is increased during labor. The average values for the entire 40 patients show an increase from a value of 35.9 mg. in early labor to 39 mg. per 100 c.c. blood immediately

TABLE I

GLUTATHIONE PER 100 C.C. OF BLOOD

| CASE NO. | LABOR | AFTER DELIVERY | FIRST DAY | THIRD DAY | FIFTH DAY | SEVENTH DAY | NINTH DAY | A. P.* WEIGHT | BLOOD LOSS | PER CENT BODY WEIGHT |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 30.7 | 35.7 | 33.5 | 35.5 | 43.2 | 38.7 | 39.1 | 57.0 | 210 | 0.39 |
| 2 | 32.3 | | 35.9 | 34.8 | 32.7 | 34.1 | 37.0 | 77.0 | 500 | 0.65 |
| 3 | 36.8 | | 41.7 | 38.4 | | 46.6 | 44.6 | 79.0 | 380 | 0.48 |
| 4 | 38.2 | 40.7 | 46.7 | 44.9 | | 50.0 | 53.0 | 70.0 | 35 | 0.05 |
| 5 | 36.8 | 35.2 | 40.2 | 35.2 | 41.9 | 41.4 | | 72.2 | 260 | 0.36 |
| 6 | 41.2 | 44.9 | 36.8 | 35.0 | 36.3 | 39.8 | 37.6 | 60.0 | 300 | 0.50 |
| 7 | 33.8 | 34.5 | 29.3 | 26.9 | 29.0 | 31.4 | | 84.0 | 910 | 1.08 |
| 8 | 28.7 | 29.4 | 25.4 | 27.7 | 27.8 | 29.8 | 31.0 | 69.0 | 300 | 0.43 |
| 9 | 34.3 | 37.2 | 35.3 | 32.1 | 32.3 | 41.4 | 36.8 | 71.7 | 500 | 0.70 |
| 10 | 33.6 | 36.1 | | 32.0 | 37.8 | 40.0 | 42.7 | 62.5 | 400 | 0.64 |
| 11 | 35.3 | 42.4 | | 39.3 | 41.2 | 51.1 | 46.7 | 72.7 | 340 | 0.47 |
| 12 | 32.9 | 31.8 | 34.1 | 32.7 | 33.6 | 41.7 | 38.9 | 61.0 | 300 | 0.49 |
| 13 | | 46.7 | 45.7 | 38.0 | 47.3 | 46.0 | 44.9 | 62.2 | 210 | 0.34 |
| 14 | 44.0 | 42.4 | 38.7 | 49.6 | 42.9 | 49.3 | 48.9 | 61.0 | 190 | 0.31 |
| 15 | 35.7 | 35.9 | 29.4 | 34.1 | 32.9 | 3[illegible] | 37.0 | 67.0 | 520 | 0.78 |
| 16 | 25.3 | 30.2 | 25.5 | 25.1 | 26.2 | 29.5 | 25.5 | 75.5 | 340 | 0.45 |
| 17 | 29.2 | 31.2 | | 27.9 | 30.4 | 33.0 | 34.5 | 76.0 | 500 | 0.75 |
| 18 | 36.6 | 37.4 | 34.6 | 28.8 | 32.5 | 36.4 | 41.4 | 47.0 | 150 | 0.32 |
| 19 | 43.5 | | 51.8 | 44.3 | 44.3 | 53.4 | 51.4 | 78.8 | 400 | 0.51 |
| 20 | 36.8 | 42.2 | 40.5 | 40.5 | 50.0 | 41.7 | 41.9 | 75.5 | 110 | 0.15 |
| Average | 35.0 | 37.3 | 36.8 | 35.1 | 36.8 | 40.7 | 41.3 | 64.6 | 199 | 0.32 |

*Ante partum.

TABLE II

GLUTATHIONE PER 100 C.C. OF BLOOD

| CASE NO. | LABOR | AFTER DELIVERY | SECOND DAY | FOURTH DAY | SIXTH DAY | EIGHTH DAY | TENTH DAY | A. P. WEIGHT | BLOOD LOSS | PER CENT BODY WEIGHT |
|---|---|---|---|---|---|---|---|---|---|---|
| 21 | 31.1 | | 29.8 | 32.5 | 34.8 | 36.4 | 42.2 | 56.5 | 650 | 1.15 |
| 22 | 30.0 | 39.3 | 40.0 | 40.7 | 42.4 | 48.9 | 46.7 | 70.6 | 120 | 0.17 |
| 23 | 34.5 | | 33.5 | 36.4 | 40.0 | 41.4 | 39.1 | | | |
| 24 | 37.0 | 41.9 | 47.9 | 42.2 | 44.0 | 51.4 | 44.9 | | | |
| 25 | 30.3 | 34.0 | 34.5 | 34.5 | 38.7 | 39.3 | 37.6 | | | |
| 26 | 49.6 | 30.8 | 30.3 | 31.7 | 36.3 | 37.0 | 36.8 | 62.7 | 250 | 0.40 |
| 27 | 35.5 | 37.4 | 36.1 | 37.4 | 36.3 | 38.9 | 38.9 | 67.6 | 90 | 0.13 |
| 28 | 34.3 | 36.8 | 30.6 | 32.5 | 35.2 | 35.9 | 41.2 | 60.3 | 150 | 0.25 |
| 29 | 43.5 | 53.8 | 40.7 | 42.8 | 48.3 | 51.8 | 54.2 | 62.5 | 410 | 0.66 |
| 30 | 29.9 | 36.1 | 32.3 | 31.8 | 33.3 | 37.6 | 36.1 | 65.1 | 60 | 0.09 |
| 31 | 34.5 | 36.3 | 37.6 | 34.8 | 43.5 | 37.8 | 38.9 | 78.6 | 100 | 0.13 |
| 32 | 33.5 | | 36.2 | 31.7 | 41.1 | 43.2 | 41.7 | 62.8 | 100 | 0.16 |
| 33 | 32.4 | 37.0 | 32.7 | 38.0 | 38.0 | 43.2 | 42.7 | 63.0 | 40 | 0.06 |
| 34 | 32.0 | 33.5 | 38.0 | 34.3 | 38.9 | 38.9 | 38.0 | 68.8 | 70 | 0.10 |
| 35 | 38.2 | 41.2 | 38.9 | 38.4 | 43.7 | 48.6 | 54.2 | 60.0 | 50 | 0.08 |
| 36 | 43.7 | 44.3 | 41.2 | 48.3 | 52.6 | 57.8 | 66.6 | 74.0 | 200 | 0.27 |
| 37 | 46.0 | 47.0 | 40.0 | 37.4 | 46.7 | 44.9 | 44.9 | 56.3 | 230 | 0.41 |
| 38 | 43.7 | 53.4 | 52.2 | 56.9 | 48.6 | 56.0 | 59.8 | 53.7 | 170 | 0.32 |
| 39 | 43.2 | 50.4 | 40.5 | 35.3 | 42.4 | 45.7 | 39.1 | 75.5 | 110 | 0.15 |
| 40 | 35.3 | 38.9 | | 32.5 | 33.3 | 35.0 | 31.7 | 63.0 | 550 | 0.87 |
| Average | 36.9 | 40.7 | 37.5 | 37.5 | 40.9 | 43.5 | 43.8 | 68.9 | 343 | 0.45 |

following delivery. This corresponds to an increase of approximately 8 per cent. Our values agree very well with the finding of Oberst and Woods, who found an increase from 35.1 to 37.6 mg. per cent for the same period. As a matter of fact the figures in Table I are practically identical with those of Oberst and Woods.

During the first and second days of the puerperium the concentration of glutathione decreases and reaches a minimum on the third day. This period of decline is followed by one of rapid increase which continues until the eighth day from which time it tends to maintain a level of between 41 and 43 mg. glutathione per 100 c.c. blood. To illustrate these changes in another manner which also compensates for the different original averages, namely, 36.9 and 35.0 mg., the average values for each day, expressed as a percentage based on the values obtained during labor as being 100 per cent, are plotted in Fig. 1. Here one can readily see that the concentration of blood glutathione is increased about 8 per cent during labor. During the first few days following delivery it

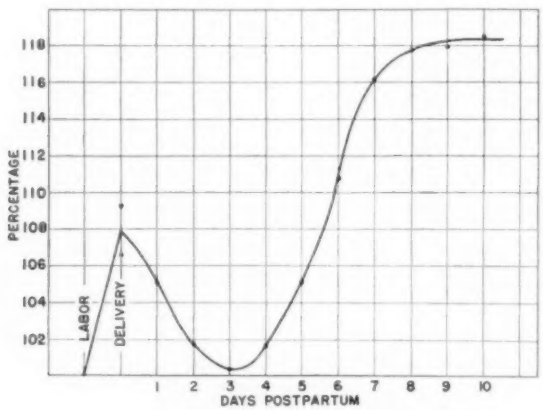


Fig. 1.

decreases until on the third day when it has approximately the same values as during early labor. The increase which then follows is rather rapid until the eighth day. From the eighth to the tenth day post partum it maintains a level approximately 18 per cent higher than that during labor.

Delivery is always associated with blood loss, and in view of the fact that bleeding affects the concentration of glutathione in the blood, the question naturally arises whether the changes observed during the first three days of the puerperium may be caused by the blood lost at delivery. On the basis of Pastore's investigation on cell volume and its relation to blood loss, in which he shows that the cell volume on the third day of the puerperium is dependent upon the amount of blood lost during delivery and the cell volume before delivery, it seems that the increase of glutathione during labor and the subsequent decrease during the first three days of the puerperium may be accounted for, at least in part, by the changes in cell volume.

A study of Table I shows that in those patients having the higher blood losses, expressed as a percentage of body weight, the content of glutathione tends to be decreased to a greater extent on the third day post partum than in those

patients having a smaller blood loss at delivery. To emphasize this we may consider two cases, one having a low and the other a high blood loss. Case 4 can be used as an illustration of the former. This patient lost only 0.05 per cent. In this instance the blood glutathione was increased during the first day. On the third day it was lower than the day preceding but, on the other hand, it was still higher than either in early labor or immediately following delivery. During the remaining days it showed a further rise. Thus except for the third day, the glutathione continued to rise from early labor until the ninth day post partum. In contrast to the above findings Case 7 has a blood loss of 1.08 per cent. In this patient we note an increase of glutathione during labor, but a decided decrease during the first day post partum. On the third day post partum the value is 26.9 mg. as compared to the value of 33.8 mg. during early labor. This is a net decrease of 20 per cent. This is identical to the change which one would expect in cell volume. All of the individual cases do not agree as well as this particular case with the concept of Pastore, but as a whole the agreement is noteworthy. This is particularly true if one considers the average glutathione values in Table I. According to Pastore the cell volume should be the same during labor and on the third day post partum if the amount of blood lost at delivery is equivalent to 0.30 per cent of the body weight. The average blood loss for the group listed in Table I is 0.32 per cent, and as already pointed out we have found the glutathione to be approximately the same during labor and the third day post partum. It is unfortunate that parallel determinations of glutathione and cell volume were not carried out so that direct comparisons could be made, but from the evidence so far obtained, it would appear that the changes during the first three days of the puerperium are closely related to changes in cell volume.

The increase from the third to eighth day, reported above, cannot be accurately interpreted in the light of our present knowledge, but it does lead one to speculate on the possibility that it is a continuation of the process which is instituted during labor or perhaps is due to the formation of new red cells which have a higher glutathione content.

## SUMMARY

A study has been made of the blood glutathione in a series of 40 patients during labor and in the puerperium. Blood was obtained from nearly all of the patients early in labor and immediately following delivery. In addition blood was drawn from 20 of these patients on the first, third, fifth, seventh, and ninth days, and from the other 20 on the second, fourth, sixth, eighth, and tenth days of the puerperium. On the basis that the values obtained early in labor represent 100 per cent, the glutathione content of the blood at delivery and in the days following has been expressed as a percentage of these values and is shown in Fig. 1.

## CONCLUSIONS

1. During labor the blood glutathione increased from an average value of 35.9 mg. in early labor to 39.0 mg. immediately folowing delivery. This is an increase of 8 per cent.

2. Following delivery there is a decrease during the first three days of the puerperium, which is probably due to bleeding incidental to delivery.

3. From the third to the eighth day post partum there is a rapid increase in the glutathione content of the blood. This may be a continuation of the process which is instituted during labor, or to the formation of new red blood cells which have a higher glutathione content.

4. Between the eighth and the tenth day of the puerperium the concentration of glutathione in the blood maintains a level approximately 18 per cent higher than that noted during early labor.

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# LABORATORY METHODS

## THE DETERMINATION OF PARA-AMINOBENZENESULFONAMIDE*

### A Micromethod

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BECAUSE the urinary excretion of sulfanilamide is dependent upon the circulation, hydration, and renal function of the individual, excretion of the drug is uncertain. Therefore, the effective use of sulfanilamide and the avoidance of untoward reactions may be dependent upon following its concentration in the blood.

To facilitate the accurate control of the concentration of sulfanilamide in the blood during treatment, the following modification of Marshall's method of analysis[1-3] is used in this clinic. As the amount required for the determination is between 0.001 and 0.015 mg. of sulfanilamide, a single 0.1 c.c. sample of capillary blood suffices for the determination of both the free and acetylated sulfanilamide in patients receiving the drug. This microprocedure has a greater accuracy than that of the original method. As reported by Marshall[3] the dimethyl-naphthylamine reagent is neither standard nor stable. The lack of uniformity of this reagent may be the source of appreciable error in the macromethod. The method reported here provides adequate control of possible error from this source.

#### METHOD

*Apparatus.*—The Evelyn photoelectric colorimeter with filter No. 520, which transmits light from 495 to 550 mμ with maximum at 518 mμ and mean at 520 mμ, is used. The maximum absorption of the characteristic color of the reaction is at 526 mμ.

*Reagents.*—Saponin, 0.05 per cent solution.

p-Toluenesulfonic acid, Eastman Kodak Co., 5 per cent solution. Unless the conjugated form of sulfanilamide is to be determined, a 5 per cent solution of trichloracetic acid may be substituted for the p-toluenesulfonic acid.

Sodium nitrite, 0.1 per cent solution, made up every few days.

Dimethyl-α-naphthylamine, Eastman Kodak Co., 1. c.c. of this solution in 250 c.c. of 95 per cent ethyl alcohol.

Sulfanilamide, 0.003 mg. per c.c. of water.

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*Procedure for Whole Blood.*—Take 0.1 c.c. of capillary blood by finger prick to a pipette calibrated to contain 0.1 c.c. Deliver this to a centrifuge tube containing 1 c.c. of a 0.05 per cent solution of saponin and rinse the pipette by drawing this solution up to the calibration mark several times. Let the blood saponin solution stand for five minutes and then add to it 3 c.c. of 5 per cent p-toluenesulfonic acid solution. Mix, let stand for five minutes and then filter through a 6.0 or 5.5 cm. filter paper. Take 2 c.c. and 1.0 c.c. of the filtrate to each of two colorimeter tubes for determination respectively of the free and total sulfanilamide, the tube to contain the latter being graduated at a volume of 10 c.c. Insert a cork with capillary opening into the tube containing the sample for total sulfanilamide determination and place this in a boiling water bath for ninety minutes, then add 1 c.c. of water and cool.

For the blank determination add to a colorimeter tube 0.5 c.c. of the saponin solution and 1.5 c.c. of the p-toluenesulfonic acid solution.

To each of the three colorimeter tubes containing the samples of filtrate and the blank sample, add 4.5 c.c. of water and 0.5 c.c. of the 0.1 per cent sodium nitrite solution. Mix and let stand at room temperature for five minutes. To each tube as near the same time as practical, add 3 c.c.* of the 0.4 per cent dimethyl-α-naphthylamine alcohol solution. Mix. Note the time. The color usually develops within five minutes. After this interval, insert the tube containing blank sample into the colorimeter and adjust the galvanometer reading to 100. Remove the tube containing blank, noting the reading of the galvanometer with no tube in place. This is the center setting. Immediately insert one after the other the tubes containing unknown samples and record the respective galvanometer readings. Again determine the center setting for the blank solution and the galvanometer readings for the unknown solutions, as described, ten and twenty minutes after the addition of the final reagent. These additional readings are important because, as noted by Marshall,[3] a reagent occasionally is encountered with which the development of the color is delayed. The procedure described assures that the full color of the reaction has developed and automatically takes care of any change in the blank during this time. Change in the blank sample with time must be cared for in some such manner as it varies with different reagents.

*Procedure for Urine.*—Dilute the urine so that the concentration of sulfanilamide is between 0.02 and 0.25 mg. per cent. The required dilution can be estimated from the dosage of sulfanilamide and the urine volume.

To 5 c.c. of the diluted urine in a colorimeter tube, add 1.5 c.c. of p-toluenesulfonic acid reagent and 0.5 c.c. of the sodium nitrite reagent.† Mix and let stand five minutes. Add 3 c.c. of the dimethyl-α-naphthylamine reagent. Mix. After five minutes, read the galvanometer deflection using the center setting from the blank tube as described in the whole blood procedure.

*Less than 3 c.c. of a reagent that acts quickly will produce full color development within the prescribed time, but with the slower acting reagents the full 3 c.c. are required. Hence, in spite of the fact that the change in the blank is proportional to the amount of reagent used, we have felt it advisable to specify 3 c.c. rather than a smaller volume.

†For the determination of total sulfanilamide the acidified urine is incubated before the addition of the nitrite solution and the total volume in the colorimeter tube adjusted to 10 c.c. after the addition of the dimethyl-α-naphthylamine solution as described in the procedure for whole blood.

*Calculation of Results.*—If the galvanometer is always adjusted to 100 with the tube containing the blank sample as directed, and if the galvanometer reading is proportional to the amount of light transmitted

$T = \frac{G}{100}$, where T is light transmission of the sample solution and G is the galvanometer reading.

Moreover, if the laws of Lambert and Beer apply with the solution and filter used

$C = \frac{1}{K} \times \log_{10} \frac{1}{T} = \frac{1}{K} \times \log \frac{100}{G} = \frac{1}{K} (2 - \log G)$, where C is the concentration of the chromogen in milligrams per cubic centimeter in the sample solution and G is the galvanometer reading for that solution. The validity of the constant and its numerical value for any colorimeter is conveniently determined by a few determinations on sample solutions containing known amounts of sulfanilamide giving galvanometer readings of from 10 to 90. Using the photoelectric colorimeter in this laboratory with three different sulfanilamide solutions from which dilutions were made and four lots of the dimethyl-α-naphthylamine reagent (see Table I), the value of K has been found to be 503 ± 8.* The validity of the constant and its numerical value should be established for each photoelectric colorimeter. Since the constancy of K in the equation depends on the full development of color, it is essential that the time required for full development of the color be checked for a given reagent every two weeks and for each new lot of reagent. In the micromethod described here change in the blank does not affect K, if the galvanometer readings for the blank and unknown samples are determined after the same time interval, as described in the procedure.

The milligrams of sulfanilamide per 100 c.c. of blood or original urine are obtained by the following equation:

milligram per cent $= \frac{100 \cdot V (2 - \log G)}{A \cdot K}$, where V = cubic centimeters of final solution in colorimeter tube and A = cubic centimeters of original blood or urine used in analysis.

## RESULTS

Our experience confirms Marshall's finding[3] that different lots of dimethyl-α-naphthylamine may provide reagents that vary in respect to both blank and time of reaction. Moreover, the same sample on standing either in the original solution or in the alcoholic reagent may undergo a change that alters its action.

Table I shows the variation in the development of color in blank solutions made with four different dimethyl-α-naphthylamine solutions. The relative speed with which the full color developed in known solutions of sulfanilamide with each of these reagents in the amount specified in the procedure is also noted in the table. Since the speed of the reaction is proportional to the amount of reagent used within certain limits, the amount of reagent to be used in the procedure was selected as that which gave full color development within five

*As a matter of convenience the concentrations of acetyl sulfanilamide have been calculated as sulfanilamide. Since the former yields 80.3 per cent of its weight as sulfanilamide,[3] sulfanilamide × 1.245 equals the acetyl compound.

minutes with the slowest acting reagent. Unnecessarily large amounts of reagent are undesirable as the change in the blank with time is also proportional to the amount used and rapidly changing blanks are a source of error.

Table II presents a comparison of analyses of bloods containing known amounts of sulfanilamide by the method of Marshall[3] and the micromethod described here. Considerable difficulty has been encountered in matching the

TABLE I

Variation in Blank at Five, Fifteen, and Thirty Minutes After Addition of Different Dimenthyl-α-naphthylamine Reagents and Relative Speed of Full Color Development.

| REAGENT | GALVANOMETER READING OF CENTER SETTING* | | | SPEED OF REACTION |
|---|---|---|---|---|
| | 5 MIN. | 15 MIN. | 30 MIN. | |
| 1 | 69.0 | 79.0 | 100.0 | Fast |
| 2 | 60.7 | 61.5 | 62.5 | Slow |
| 3 | 70.7 | 84.0 | (100+) | Fast |
| 4 | 61.0 | 62.0 | 62.3 | Slow |

*The center setting is the galvanometer reading obtained when the test tube containing the blank sample is removed from the colorimeter after the galvanometer reading has been adjusted to 100 with the blank sample tube in place (see procedure).

known and unknown solutions by visual colorimetry without filters when using a reagent that develops a large blank. Apparently some secondary color develops at different rates in the two solutions when their concentrations are not the same. Transmission curves on such samples have shown absorption bands at both 400 mμ and 600 mμ. These two bands are beyond the transmission limits of the filter used with the photoelectric colorimeter and therefore do not inter-

TABLE II

Comparison of Analyses by Macrovisual Colorimetry Method[3] and by Microphotoelectric Colorimetry Method of Whole Blood to Which Known Amounts of Sulfanilamide Had Been Added.

| KNOWN SULFANILAMIDE MG. PER CENT WHOLE BLOOD | REAGENT NO.* | MACROMETHOD[3]† | | | MICROMETHOD | |
|---|---|---|---|---|---|---|
| | | COLORIMETER READING | MG. PER CENT FOUND | PER CENT ERROR | MG. PER CENT FOUND | PER CENT ERROR |
| 4.76 | 4 | Too high | | | 4.73 | −0.6 |
| 4.76 | 1 | Too high | | | 4.67 | −2.5 |
| 9.09 | 4 | 29.9 | 10.04 | − 9.0 | 9.05 | −0.5 |
| 9.09 | 1 | 23.5 | 12.80 | +28.9 | 9.13 | +0.5 |
| 16.67 | 4 | 19.2 | 15.60 | − 6.3 | 16.58 | −0.5 |
| 16.67 | 1 | 16.9 | 17.76 | + 5.7 | 16.70 | +0.1 |
| 23.08 | 4 | 13.9 | 21.60 | −10.7 | 23.10 | +0.1 |
| 23.08 | 1 | 12.5 | 24.00 | + 3.7 | 22.92 | −0.7 |

*Numbers correspond with those used in Table I.
†Standard of 1.5 mg. per cent set at 20.0.

fere in the procedure described here. Such an interpretation is supported by the fact that a photoelectric colorimeter determination of the sulfanilamide concentration of a 1:20 dilution of a macrofiltrate that is difficult to read gives results that check with the microanalyses and the known concentrations.

Table III gives the results of analyses by the method described here of free and acetyl sulfanilamide in whole blood containing known added amounts of the free and acetyl compounds.

TABLE III

Recovery by the Microprocedure of Free and Acetylated Sulfanilamide from Whole Blood Containing Known Added Amounts of the Free and Acetylated Compounds, as Milligrams Per Cent.

| SAMPLE | FOUND ACETYL SULFANILAMIDE | THEORETICAL ACETYL SULFANILAMIDE | FOUND FREE SULFANILAMIDE | THEORETICAL FREE SULFANILAMIDE |
|---|---|---|---|---|
| Whole blood 1 | 3.84 | 3.82 | 0 | 0 |
| Whole blood 1 | 3.94 | 3.82 | 0 | 0 |
| Whole blood 1 | 3.68 | 3.82 | 0 | 0 |
| Whole blood 2 | 3.35 | 3.65 | 6.84 | 6.82 |
| Whole blood 2 | 3.67 | 3.65 | 6.77 | 6.82 |
| Whole blood 2 | 3.67 | 3.65 | 6.77 | 6.82 |

SUMMARY

A modification of Marshall's method for the determination of both the concentration of free and total sulfanilamide in whole blood requiring but 0.1 c.c. of capillary blood is described.

Errors involved in this procedure and the macroprocedure of Marshall[3] are described and discussed.

The micromethod described here is less susceptible to error than the macroprocedure.

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# OBSERVATIONS ON THE NEUFELD REACTION (QUELLUNG TEST) FOLLOWING DESICCATION OF PNEUMOCOCCUS PREPARATIONS

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DURING the performance of routine examination of sputum and cultures for specific types of pneumococci some observations were made that may be of considerable practical importance to laboratory workers and to physicians. Loss of time in detecting the presence of a specific type pneumococcus can be avoided, and distance from a laboratory because of the time required to deliver the specimen to the pathologist or laboratory worker will prove no obstacle for the danger of destruction or damage by capsular change to the specimen by autolysis or contaminating overgrowth of other bacteria may be eliminated by use of the method of preparation of dried smears as described. Also dried preparations offer a method of collecting pneumococcus type material for teaching purposes in the student classroom because of the indestructibility of capsular substance.

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Neufeld[1] in 1902 observed and reported the reaction of specific antiserum on capsules of pneumococci. In 1931 Neufeld[2] and several others developed the method of identification of the types of different pneumococci by noting the swelling of the capsules when mixed with a specific antiserum. This method is now in extensive diagnostic use for type pneumococcal identification because of the simplicity, rapidity, and reliability of the test.

In the observations reported in this paper the material used consisted of film preparations of sputum and cultures containing pneumococci of known types. These were dried, some were examined immediately, some were frozen with carbon dioxide gas, some were placed in alcohol and ether mixture for varying periods of time, others were placed in a hot air chamber at high temperatures for several months, and still others were covered with paraffin at 75° C. for a few weeks and then washed in xylol, ether, and alcohol before examination. Another departure from the usual procedures was to place on microscopic

Fig. 1.—Pneumococcus preparation kept at 60° C. for one year dried on microscopic slide.

slides a small amount of specific type serum, such as Types I, II, III, etc., and dry either at room or incubator temperature. When it was desired to test a specimen of sputum or culture, a drop of such material to be tested was placed on each dried specific type serum, methylene blue stain was added to the preparation, a cover slip was placed over the mixture, and examination was made. Positive Neufeld reactions were obtained in all cases after exposure as is shown below. Whether it is coincidental or not is not known, but in the specimens examined there was encountered no delay in the development of the capsular swelling as is sometimes found in some specimens of sputum that may require as much as half an hour before the phenomenon develops.

The several tests made on the capsular substance and pneumococci are as follows:

1. A test was made to determine the effect of freezing with carbon dioxide gas to see if crystallization of the capsule and subsequent alteration in its structure could be produced. After this treatment, the preparation was allowed to

stand at room temperature for a time to allow any changes that might be possible to take place, and then specific type serum and dye were added and examination was made. A typical swelling of the capsule developed, and a definite type diagnosis could be made on the pneumococci in the preparation.

2. Next exposure of the dried sputum spread to the action of mixture of alcohol and ether was made, and on examination the capsules of the specific types of pneumococci promptly developed.

3. Sputum films were dried and examined after different periods of time, ranging from a few minutes to days, weeks, and months, and when type specific serum and methylene blue was applied, capsular swelling of the specific type of pneumococci developed at once.

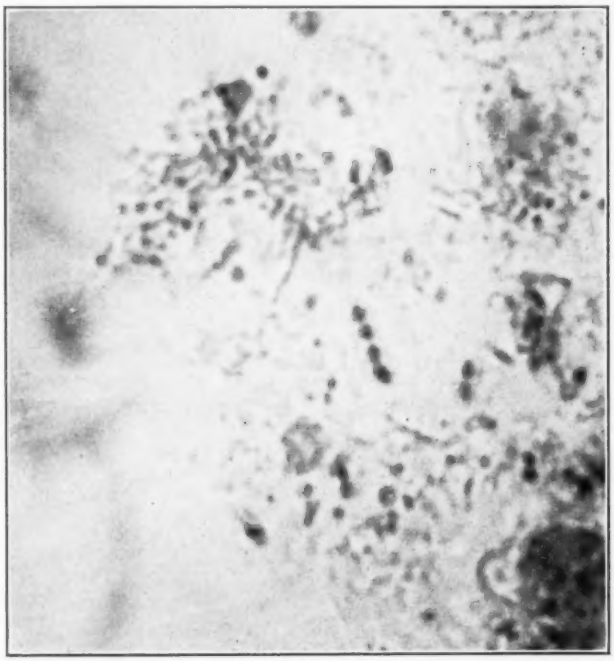

Fig. 2.—Pneumococcus preparation dried on slide and kept in frozen state in ice for one year.

4. Dried sputum smears containing known types of pneumococci were placed in a hot paraffin oven at 55° C. for four months and then examined for capsular swelling. Successful results were obtained in all specimens.

5. Dried sputum preparations containing known types of pneumococci were kept for over twelve months at a temperature of 60° C., and on the application of the type specific serum and the stain, positive Quellung test was obtained.

6. Specimens of sputum of known types were dried on cover slips and some of the type specific serum was dried on microscopic slides. A drop of Löffler's methylene blue was added to the sputum film, and the cover slip with the sputum film was placed on the dried specific type serum. The capsules of the type specific pneumococci promptly developed in the usual way.

7. Dried sputum specimens on microscopic slides were kept at 75° C. immersed in paraffin for several weeks. The paraffin was removed with xylol,

washed with ether, then alcohol, dried, and stained with methylene blue. When the specific type serum was added, a positive Quellung test promptly developed.

8. Another exposure to unusual conditions was to store preparations in ice frozen in to the blocks in a frigidaire unit which from time to time was defrosted and then frozen again. After one year of such exposure, the preparations when tested gave positive Quellung reactions. These Quellung preparations may be indefinitely preserved by painting the margins of the cover slip with Duco paint.

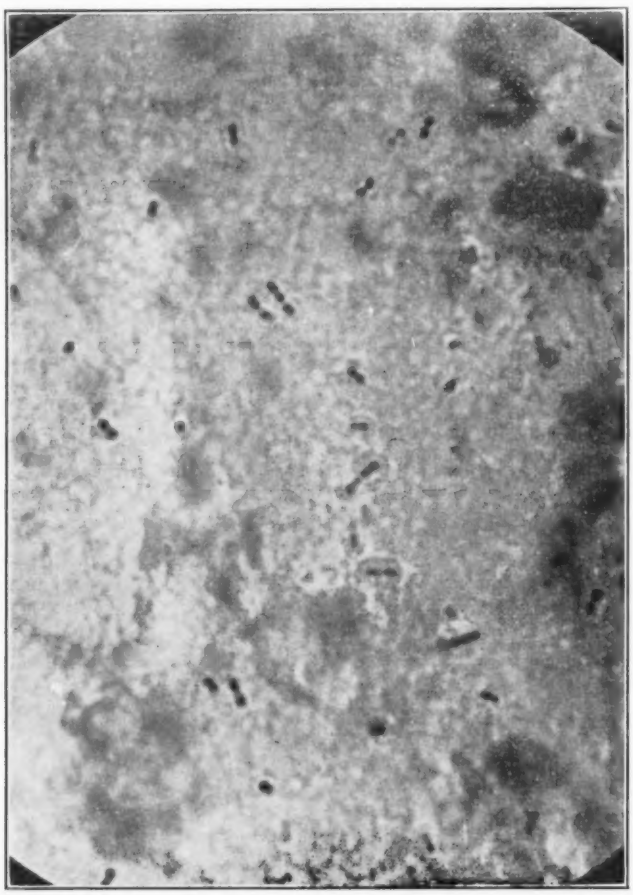

Fig. 3.—Pneumococcus preparation dried and kept for one year at room temperature. Photostat with maximum contrast.

Time seems to make the reaction more pronounced, for the capsules swell to a greater thickness and appear sharper in outline.

The dried preparations insure a constant type, that is, transmutation from one type to another is impossible under the changed conditions produced by the desiccation. Transmutation of one type to another was shown to take place by Griffith in 1928, and Neufeld confirmed this observation in the same year. Dawson and Sia in 1931 and others proved that pneumococci of one type may revert to another type of this class.

These observations direct our attention to the bacterial cell and to the composition of the capsule to explain the property of water attraction or the absence there of the so-called hydrophile or hydrophobe property. That bacteria have an endoplasm and an ectoplasm in zonal relationship at least is fairly universally accepted. From Wamoscher's studies on typhoid and colon bacilli and other forms it was developed that the bacterial cells have a membrane of great elasticity, encasing a colloidal sol that under certain conditions may assume the form of gel.

As to the origin of the capsule many think it is formed by change in consisteney of the outer layer of the bacterial cell. Others (Meyer and Zettnow) think it is an active secretion. Bacteria also secrete mucoid substance and some (anthrax bacilli) form capsules of protein substance, but the pneumococcus has on the surface a mixture of components consisting of polysaccharides, nitrogen, phosphorus, and lipoidal substance. The central sol or protoplasmic substance has no species or type specific property, but in the capsular components is a type specific antigenic component.

Considerable work has been done to determine the chemical nature of the capsular substance and much has been accomplished in this direction through the work of Avery and Heidelberger,[3] Morgan and Avery,[4] Cooper,[5] Heidelberger and Goebel,[16] and others. When isolated by extraction and precipitation in a chemically pure state, the capsular substance was found to be made up chiefly of components of complex polysaccharides. Of the components of the polysaccharides, galacturonic acid, amino-sugar derivative, glucose, and glucuronic acid have been isolated. Wadsworth and Brown in 1931 reported a specific antigenic component in Type I pneumococci that differed from the specific capsular polysaccharide prepared, and Avery and Goebel (1933) determined this component to be an acetylated form of Type I capsular polysaccharide. It is suggested that it is in this form that it exists in the normal bacterium. By extraction and precipitation certain antigenic properties are lost by the removal of acetyl groups, and there remains a deacetylated polysaccharide that is specific for Type I pneumococci. Tillett, Goebel, and Avery in 1930 isolated another component that yields about 30 per cent reducing sugar on hydrolysis, contains phosphoric acid, and 6.1 per cent of nitrogen and is not type specific. With pneumococci the change from the S to the R variant is associated with an increase in the lipoidal component of the surface of the latter form and a decrease in the polysaccharide component. Lipides are derived from carbohydrates probably by a process of reduction that removes oxygen or hydroxyl and substitutes hydrogen for it.

Among the polysaccharides are found starch, dextrin, glycogen, inulin, pectin, humin, cellulose, gums, and vegetable mucilages. In most substances polysaccharides do not form crystals. Water dissolves some, some are unaffected by water, some are insoluble in cold water but are soluble in hot water. Diastases are specific for certain polysaccharides only and are inert against others. The pneumococcus capsule may contain phospholipins which are amorphous substances, white or cream colored when pure. They are very hygroscopic and rapidly attract water. They are sparingly soluble in mixtures of alcohol and ether, but some may dissolve in one or the other. They may be dried without

decomposition in very low temperatures and in vacuo, or in a different atmosphere such as some indifferent gas. The hydrophilic property of the capsule or the capsular affinity for water when acted upon by a catalyzer is probably due to the component parts of the polysaccharide and to the lipides especially. These observations may prove of value in making possible simple diagnostic units that so far have been shown to be completely reliable. It is possible to dry the specific types of serum on slides and use at any time that is desired, thus offering a simpler method as compared with the method now in use of distribution in vials or capillary tubes.

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## ERRORS IN ERYTHROCYTE COUNTS DUE TO HAYEM'S SOLUTION—AVOIDED WITH GOWERS' SOLUTION*

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AN IDEAL diluting fluid for the enumeration of erythrocytes should not hemolyze the corpuscles, should promote their even distribution without clumping, should not bring about gross precipitation of proteins or other substances in the blood, should act as a fixative so that the cells retain essentially their original shape, and should prevent growth of bacteria and molds.

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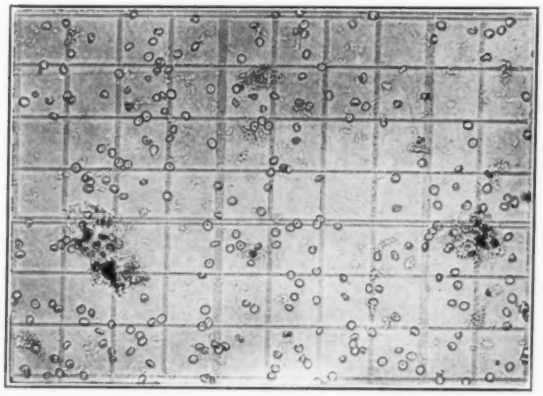

Fig. 1.—Photograph of counting chamber (×180) showing precipitated material and clumps in capillary blood from a patient with kala azar. Hayem's solution used as diluent.

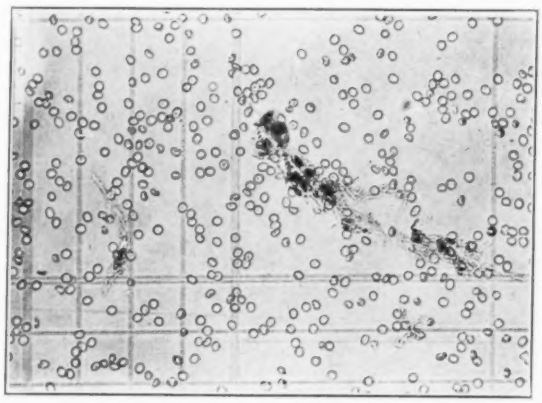

Fig. 2.—Photograph of counting chamber (×180) showing clumping of red corpuscles from the capillary blood of a patient with lobar pneumonia. Hayem's solution used as diluent.

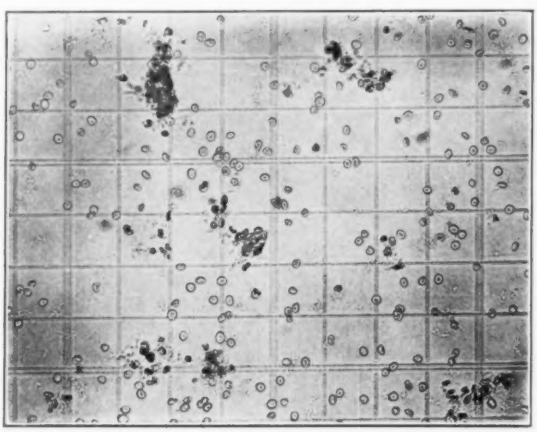

Fig. 3.—Photograph of counting chamber (×180) showing clumping of cells in oxalated venous blood from a patient with kala azar. Hayem's solution used as diluent.

During the course of studies of the blood of patients with nephritis, cirrhosis of the liver, kala azar, and other diseases, it was observed in many instances that when erythrocyte counts were made with Hayem's solution there appeared immediately, in the blood pipette and in the counting chambers, coarse precipitated particles of irregular size which did not disappear after vigorous and prolonged shaking of the pipette (Figs. 1 to 3). This precipitate, when examined under the microscope, consisted of an amorphous translucent material to which adhered many red blood corpuscles. The result was an uneven distribution of erythrocytes in the chamber. On account of the coarseness of the precipitate there also occurred rapid sedimentation in the pipette, preventing the delivery to the chamber of a true sample of its contents. Because of these difficulties it was found, as will be shown later, that the error was very great in the actual enumeration of erythrocytes and hence in the calculation of the color index, the mean corpuscular volume and the mean corpuscular hemoglobin in many of the

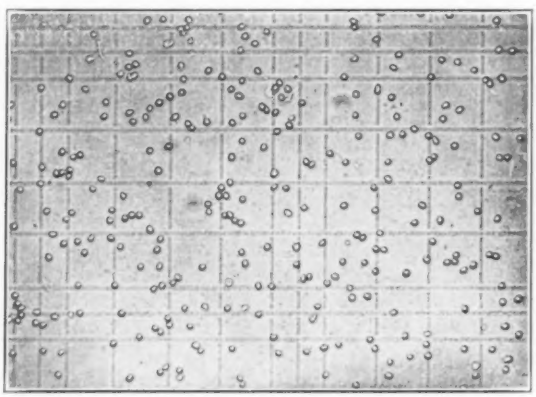

Fig. 4.—Photograph of counting chamber (×180) showing no precipitate in oxalated venous blood from a patient with kala azar. Gowers' solution used as diluent.

cases studied. A search was made, both in the medical literature and by means of experiments, for other diluting fluids which would give more satisfactory results.

## REVIEW OF LITERATURE

A large number of diluting fluids have been proposed but all are subject to one or more objections. The solution devised by Hayem[1, 2] in 1878 is almost universally used and is recommended as the best fluid by the authors of most of the standard text and reference books.[3-6] A few workers have noted difficulties with the use of this fluid. Hayem[2] himself considered the use of his diluent unsatisfactory when the "fibrin" content of blood was increased. He observed, in some cases, a precipitation of fine particles which he thought were composed of "albuminoid principles." Jacobson[7] cited a case of multiple myeloma in which there occurred an abundant white precipitate when the blood was diluted with Hayem's solution. Reimann,[8] Bönniger,[9] and Foord,[10, 11] observed similar phenomena in their cases. Reitmann,[12] Klein,[13] and Clough[14] noted clumping of the red blood corpuscles in cases of multiple myeloma, cirrhosis of the liver and pneumonia, but it was not stated in their papers what diluting fluid was used.

In some instances it was believed that autoagglutination was responsible for the clumping.

### DILUTING FLUIDS STUDIED

Solution of chemically pure sodium chloride (0.85 per cent) usually gave satisfactory results when the counts were made immediately with very carefully cleaned apparatus and when great care was used in the preparation of the solution with distilled water which was strictly neutral in reaction. Occasionally, even with these precautions, hemolysis occurred in the counting chamber and introduced error. Owing to this fact and because isotonic solution of sodium chloride does not fix or preserve the erythrocytes, it is believed that it cannot be used with safety in clinical work as a substitute for other diluents.

TABLE I

AMOUNT OF PRECIPITATE AND DEGREE OF CLUMPING OF ERYTHROCYTES WHEN BLOOD OR SERUM FROM NORMAL PERSONS AND FROM PATIENTS WITH KALA AZAR IS ADDED TO VARIOUS DILUENTS

| DILUENT | FORMULA OF DILUENT | | PRECIPITATE IN SERUM DILUTED 1:200 | | R.B.C. CLUMPING IN OXALATED BLOOD DILUTED 1:200 | | REMARKS |
|---|---|---|---|---|---|---|---|
| | | | KALA AZAR | NORMAL PERSON | KALA AZAR | NORMAL PERSON | |
| Hayem's | Sodium chloride | 1.0 | ++++ | ± | ++++ | ± | |
| | Sodium sulfate | 5.0 | | | | | |
| | Mercury bichloride | 0.5 | | | | | |
| | $H_2O$ | 200.0 | | | | | |
| Hayem's without sodium sulfate | Sodium chloride | 1.0 | ++++ | ± | ++++ | ± | |
| | Mercury bichloride | 0.5 | | | | | |
| | $H_2O$ | 200.0 | | | | | |
| Hayem's without bichloride of mercury | Sodium chloride | 1.0 | 0 | 0 | 0 | 0 | Growth of molds after a few weeks |
| | Sodium sulfate | 5.0 | | | | | |
| | $H_2O$ | 200.0 | | | | | |
| Jorgensen's | Sodium chloride | 1.0 | ++++ | ± | ++++ | ± | |
| | Sodium sulfate | 5.0 | | | | | |
| | Mercury bichloride | 0.1 | | | | | |
| | $H_2O$ | 200.0 | | | | | |
| Gowers' (formula given by Mallory and Wright) | Sodium sulfate | 12.5 | 0 | 0 | 0 | 0 | Fine precipitate after a few minutes |
| | Acetic acid | 33.3 | | | | | |
| | $H_2O$ | 200.0 | | | | | |
| Toisson's | Neutral glycerin | 3.75 | 0 | 0 | 0 | 0 | Pipette not easily cleaned |
| | Sodium sulfate | 10.00 | | | | | |
| | Sodium chloride | 1.25 | | | | | |
| | Methyl violet | 0.03 | | | | | |
| | $H_2O$ | 200.00 | | | | | |
| Isotonic saline | Sodium chloride | 1.70 | 0 | 0 | 0 | 0 | Hemolysis often occurs |
| | $H_2O$ | 200.00 | | | | | |

Various isotonic solutions of sodium fluoride, sodium benzoate, borax, and sodium thiosulfate were tried, but were discarded. Different concentrations of metaphen, merthiolate, and mercury phenyl nitrate at various pH values were tested. None of these solutions was satisfactory since they either brought about

hemolysis, coagulation, precipitation, or permitted the growth of molds or bacteria in the diluting fluids. Of all the solutions tried, that of Gowers, as given by Mallory and Wright,[15] seemed to be most satisfactory (Fig. 4). The formula for Gowers' solution, as given by Mallory and Wright and as recorded in Table I of this paper, was employed in our studies. This formula differs materially from that originally recommended by Gowers.[16] Other solutions are listed in the table and their properties with regard to precipitation and clumping are recorded.

TABLE II

NUMBER OF CLUMPS OF RED CORPUSCLES IN THE WHOLE RULED AREA OF THE COUNTING CHAMBER WHEN CAPILLARY AND OXALATED VENOUS BLOOD OF SELECTED PATIENTS WERE DILUTED WITH HAYEM'S AND GOWERS' SOLUTIONS

| SERIES NO. | DIAGNOSIS | HAYEM'S SOLUTION | | GOWERS' SOLUTION | |
|---|---|---|---|---|---|
| | | OXALATED VENOUS BLOOD* | CAPILLARY BLOOD | OXALATED VENOUS BLOOD* | CAPILLARY BLOOD |
| 1 | Kala azar | 25 | 20 | 0 | 0 |
| 2 | Kala azar | 33 | 24 | 0 | 0 |
| 3 | Kala azar | 43 | 41 | 0 | 0 |
| 4 | Kala azar | 36 | 33 | 0 | 0 |
| 5 | Kala azar | 1 | 1 | 0 | 0 |
| 6 | Chronic nephritis | 0 | 0 | 0 | 0 |
| 7 | Chronic nephritis | 0 | 0 | 0 | 0 |
| 8 | Chronic nephritis | 2 s | 1 s | 0 | 0 |
| 9 | Chronic nephritis | 21 | 12 | 0 | 0 |
| 10 | Chronic nephritis | 8 | 2 | 0 | 0 |
| 11 | Heart failure due to rheumatic c.v. disease | 2 s | 0 | 0 | 0 |
| 12 | Heart failure due to rheumatic c.v. disease | 2 s | 1 s | 0 | 0 |
| 13 | Heart failure due to hypertensive c.v. disease | 0 | 0 | 0 | 0 |
| 14 | Heart failure due to hyperthyroidism | 0 | 0 | 0 | 0 |
| 15 | Heart failure due to hyperthyroidism | 6 | 2 | 0 | 0 |
| 16 | Carcinoma of stomach | 0 | 0 | 0 | 0 |
| 17 | Carcinoma of stomach | 0 | 0 | 0 | 0 |
| 18 | Carcinoma of bile duct | 11 | 6 | 0 | 0 |
| 19 | Cirrhosis of liver | 10 | 3 | 0 | 0 |
| 20 | Cirrhosis of liver | 18 | 5 | 0 | 0 |
| 21 | Tuberculosis of mesenteric lymph nodes | 6 | 3 | 0 | 0 |
| 22 | Pleurisy with effusion and ascites, tuberculous | 0 | 0 | 0 | 0 |
| 23 | Lobar pneumonia | 10 | 6 | 0 | 0 |
| 24 | Schistosomiasis | 0 | 0 | 0 | 0 |

*2 mg. potassium oxalate and 3 mg. ammonium oxalate to 5 c.c. venous blood.
s = small clumps c.v. = cardiovascular

TECHNIQUE EMPLOYED

Having demonstrated that Hayem's solution was unsatisfactory in studies of red blood cells of various types of patients, it was decided to compare this solution with Gowers' solution and with isotonic solution of sodium chloride. For each specimen of blood to be counted two pipettes were used and two counts of the erythrocytes were made with each of the three solutions to be tested. Each erythrocyte count was performed by counting five of the medium sized squares (each medium sized square equals $\frac{1}{25}$ sq. mm.). Each of these five medium sized squares consisted of sixteen small squares (each small square equals $\frac{1}{400}$ sq.

mm.). Thus four complete red blood cell counts were made on each specimen of blood with each of the three fluids. In order to study the variation of distribution in the counting chamber the counts were recorded in terms of each medium sized square ($\frac{1}{25}$ sq. mm.). Hence for each case and with each fluid there were twenty figures for comparison. All pipettes, counting chambers, and cover slips were certified by the United States Bureau of Standards. All counts were done by one person and each pipette was shaken with a uniform rate for three minutes by hand. In some instances oxalated venous blood was employed for comparison (see Table II) but usually the capillary blood taken from the lobe of the ear was used.

### TYPES OF CASES STUDIED

Many observations were made on normal individuals and on patients suffering from a variety of disorders. For purposes of clearness and brevity it was

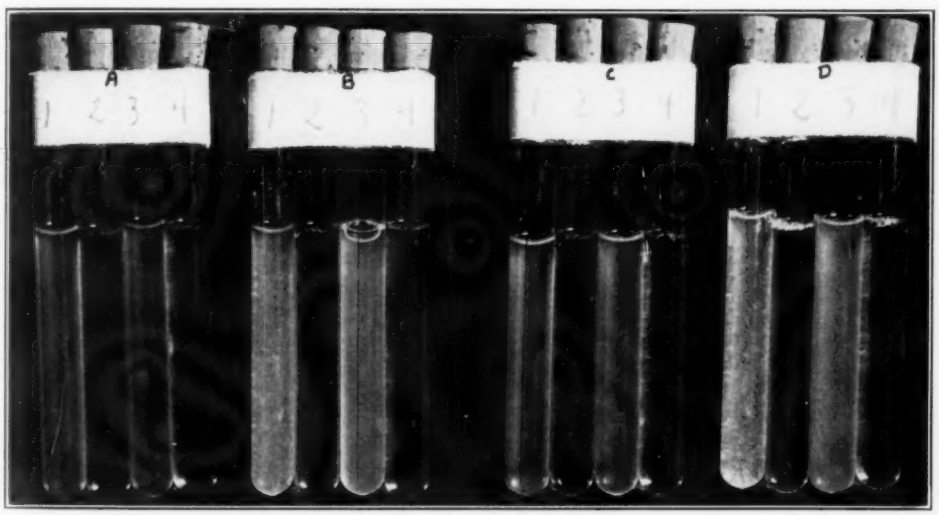


Fig. 5.—Photograph of test tubes containing mixtures of various diluents with plasma (1 part plasma to 200 parts diluent) from blood of a normal person and of a patient with kala azar. The sets of tubes labeled A and B were inverted after the mixtures had been prepared for twenty-four hours and sets labeled C and D were inverted after the mixtures had been prepared for less than one hour. Note heavier precipitate in tubes B1 and B3 than in D1 and D3. Note coarseness of precipitate in B1 and D1.

A and C
- 1 = Normal plasma + Hayem's solution
- 2 = Normal plasma + Hayem's solution without bichloride of mercury
- 3 = Normal plasma + Gowers' solution
- 4 = Normal plasma + 0.85 per cent solution of sodium chloride

B and D
- 1 = Kala azar plasma + Hayem's solution
- 2 = Kala azar plasma + Hayem's solution without bichloride of mercury
- 3 = Kala azar plasma + Gowers' solution
- 4 = Kala azar plasma + 0.85 per cent solution of sodium chloride

believed adequate to report studies on three groups of cases only. Nine patients (Table IIIA) were regarded as a control group since their blood when diluted with Hayem's solution reacted as normal. There was relatively slight precipitate and there was almost no tendency for clumping of erythrocytes in these cases. The second group comprised 16 unselected cases of kala azar (Table IIIB) and the third group contained two patients with chronic nephritis (Table IIIB) who exhibited the nephrotic syndrome. Other patients with cirrhosis of the liver, un-

TABLE IIIA

STATISTICAL ANALYSIS OF THE ERYTHROCYTE COUNTS OF THE BLOOD OF 9 CONTROL INDIVIDUALS IN WHICH CLUMPING OF THE CELLS WAS NOT MARKED

| CASE NO. | MEAN* | | | STANDARD DEVIATION | | | COEFFICIENT OF VARIATION | | | DIFFERENCE OF THE MEANS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | $M_H$-$M_G$ | $M_H$-$M_S$ | $M_G$-$M_S$ |
| 1 | 57.30±0.76 | 58.60±0.97 | 59.90±0.89 | 5.03±0.54 | 6.41±0.68 | 5.91±0.63 | 8.78±0.94 | 10.94±1.17 | 9.86±1.05 | -1.30±1.23 | -2.60±1.17 | -1.30±1.32 |
| 2 | 70.00±0.77 | 72.00±0.93 | 72.00±0.78 | 5.11±0.54 | 6.20±0.66 | 5.20±0.55 | 7.30±0.78 | 8.61±0.92 | 7.22±0.77 | -2.00±1.21 | -2.00±1.10 | 0 ±1.22 |
| 3 | 77.55±1.01 | 77.25±0.82 | 77.05±1.13 | 6.73±0.72 | 5.46±0.58 | 7.51±0.80 | 8.68±0.93 | 7.07±0.76 | 9.74±1.04 | 0.30±1.30 | 0.30±1.52 | 0.20±1.21 |
| 4 | 87.90±0.73 | 84.10±0.88 | 84.45±1.27 | 4.82±0.51 | 5.86±0.62 | 8.40±0.90 | 5.48±0.58 | 6.96±0.75 | 9.95±1.07 | 3.80±1.14 | 3.45±1.46 | -0.35±1.54 |
| 5 | 111.05±1.28 | 104.85±0.90 | 105.25±1.23 | 8.48±0.90 | 5.98±0.64 | 8.16±0.87 | 7.64±0.82 | 5.70±0.61 | 7.76±0.83 | 6.20±1.56 | 5.80±1.78 | -0.40±1.52 |
| 6 | 76.90±1.12 | 77.85±0.99 | 79.40±0.75 | 7.45±0.79 | 6.59±0.70 | 4.95±0.53 | 9.69±1.04 | 8.46±0.91 | 6.24±0.67 | -0.95±1.49 | -2.50±1.35 | -1.55±1.24 |
| 7 | 61.65±1.00 | 64.35±1.04 | 61.50±0.94 | 6.61±0.70 | 6.89±0.73 | 6.22±0.66 | 10.73±1.16 | 10.71±1.15 | 10.12±1.08 | -2.70±1.44 | 0.15±1.37 | 2.85±1.40 |
| 8 | 80.20±0.78 | 80.60±0.92 | 80.80±1.06 | 5.14±0.55 | 6.07±0.65 | 7.03±0.75 | 6.41±0.68 | 7.53±0.81 | 8.70±0.93 | -0.40±1.21 | -0.60±1.32 | -0.20±1.40 |
| 9 | 67.05±0.79 | 66.10±0.81 | 66.70±0.92 | 5.22±0.56 | 5.37±0.57 | 6.11±0.65 | 7.78±0.83 | 8.13±0.87 | 9.16±0.98 | 0.95±1.13 | 0.35±1.21 | -0.60±1.22 |
| Average | 76.62±3.40 | 76.19±2.88 | 76.34±2.93 | 6.06±0.28 | 9.04±0.10 | 6.61±0.26 | 8.05±0.34 | 8.23±0.36 | 8.75±0.29 | 0.43±0.61 | 0.28±0.59 | 0.15±0.27 |

*Mean count of erythrocytes in 20 areas of 0.4 sq. mm.

TABLE IIIB

STATISTICAL ANALYSIS OF ERYTHROCYTE COUNTS OF THE BLOOD OF 16 PATIENTS WITH KALA AZAR AND 2 WITH CHRONIC NEPHRITIS IN WHICH THERE WAS MARKED CLUMPING OF THE CELLS

| CASE NO. | MEAN | | | STANDARD DEVIATION | | | COEFFICIENT OF VARIATION | | | DIFFERENCE OF THE MEANS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | HAYEM'S SOLUTION | GOWERS' SOLUTION | 0.85% SODIUM CHLORIDE | $M_H$-$M_G$ | $M_H$-$M_S$ | $M_G$-$M_S$ |
| *Kala Azar* | | | | | | | | | | | | |
| 1 | 56.85±1.81 | 65.40±0.98 | 65.45±0.72 | 12.03±1.28 | 6.49±0.69 | 4.75±0.51 | 21.17±2.36 | 9.92±1.07 | 7.26±0.76 | - 8.55±2.06 | - 8.60±1.95 | -0.05±1.22 |
| 2 | 37.75±0.90 | 51.30±0.88 | 51.45±0.81 | 5.97±0.64 | 5.87±0.62 | 5.34±0.57 | 15.82±1.73 | 11.43±1.23 | 10.38±1.12 | -13.55±1.26 | -13.70±1.21 | -0.15±1.20 |
| 3 | 50.50±1.36 | 57.85±0.55 | 58.55±0.60 | 8.99±0.96 | 3.66±0.39 | 3.96±0.42 | 17.81±1.94 | 6.33±0.68 | 6.76±0.72 | - 7.35±1.47 | - 8.05±1.49 | -0.70±0.81 |
| 4 | 62.95±1.36 | 73.15±0.78 | 71.55±0.67 | 9.02±0.96 | 5.16±0.55 | 4.43±0.47 | 14.33±1.56 | 7.05±0.76 | 6.19±0.66 | -10.20±1.57 | - 8.60±1.52 | 1.60±1.03 |
| 5 | 47.45±1.36 | 59.10±0.66 | 57.45±0.59 | 9.03±0.96 | 4.41±0.47 | 3.92±0.42 | 19.03±2.10 | 7.47±0.80 | 6.82±0.73 | -11.65±1.51 | -10.00±1.48 | 1.65±0.88 |
| 6 | 48.85±1.61 | 64.55±0.96 | 62.85±0.75 | 10.67±1.14 | 6.34±0.68 | 4.99±0.53 | 21.85±2.46 | 9.83±1.08 | 7.94±0.86 | -15.70±1.87 | -14.00±1.78 | 1.70±1.22 |
| 7 | 47.00±1.19 | 54.05±0.64 | 53.90±0.67 | 7.88±0.84 | 4.22±0.45 | 4.44±0.47 | 16.77±1.86 | 7.82±0.86 | 8.23±0.87 | - 7.05±1.35 | - 6.90±1.37 | 0.15±0.93 |
| 8 | 52.60±1.41 | 60.75±0.69 | 59.70±0.82 | 9.36±1.00 | 4.55±0.48 | 5.46±0.58 | 17.79±1.96 | 7.49±0.80 | 9.15±0.98 | - 8.15±1.57 | - 7.10±1.63 | 1.05±1.07 |
| 9 | 56.60±1.11 | 62.55±0.86 | 63.35±0.81 | 7.37±0.79 | 5.73±0.61 | 5.36±0.57 | 13.02±1.41 | 9.16±0.98 | 8.45±0.91 | - 5.95±1.40 | - 6.75±1.37 | -0.80±1.18 |
| 10 | 54.70±0.88 | 62.10±1.20 | 59.75±0.77 | 5.86±0.62 | 7.93±0.85 | 5.10±0.54 | 10.72±1.16 | 12.77±1.38 | 8.53±0.92 | - 7.40±1.49 | - 5.05±1.17 | 2.35±1.42 |
| 11 | 68.35±1.40 | 77.35±0.87 | 80.05±0.85 | 9.26±0.99 | 5.77±0.62 | 5.65±0.60 | 13.55±1.46 | 7.46±0.80 | 7.06±0.76 | - 9.00±1.65 | -11.70±1.64 | -2.70±1.22 |
| 12 | 57.30±0.76 | 60.75±0.91 | 61.90±0.87 | 5.03±0.54 | 6.02±0.64 | 5.77±0.62 | 8.78±0.91 | 9.92±1.07 | 9.32±1.00 | - 3.45±1.19 | - 4.60±1.16 | -1.15±1.26 |
| 13 | 46.70±1.50 | 49.80±0.70 | 50.90±0.72 | 9.92±1.05 | 4.66±0.50 | 4.80±0.51 | 21.23±2.34 | 9.27±0.99 | 9.44±1.01 | - 3.10±1.66 | - 4.20±1.66 | -1.10±1.00 |
| 14 | 76.80±1.26 | 75.90±0 82 | 77.90±0.58 | 8.37±0.89 | 5.43±0.58 | 3.86±0.41 | 10 90±1.16 | 7.15±0.76 | 4.95±0.54 | 0.90±1.50 | - 1.10±1.39 | -2.00±1.00 |
| 15 | 50.85±1.24 | 49.90±0.72 | 48.95±0.76 | 8.25±0 88 | 4.78±0.51 | 5.01±0.53 | 16.22±1.71 | 9.59±1.03 | 10 24±1.10 | 0.95±1.43 | 1.90±1.45 | 0.95±1.05 |
| 16 | 75.10±1.10 | 75.65±0.70 | 75.80±0.62 | 7.27±0.77 | 4.61±0.49 | 4.13±0.44 | 9.67±1.04 | 6.09±0.64 | 5.45±0.58 | - 0.55±1.30 | - 0.70±1.26 | 0.15±0.94 |
| Average | 55.65±1.75 | 62.51±1.49 | 62.47±1.57 | 8.39±0.30 | 5.35±0.17 | 4.81±0.10 | 15.54±0.69 | 8.67±0.30 | 7.88±0.05 | - 6.86±0.79 | - 6.82±0.73 | 0.04±0.23 |
| *Chronic Nephritis* | | | | | | | | | | | | |
| 1 | 47.45±1.36 | 58.05±0.68 | 58.50±0.60 | 9.03±0.96 | 4.49±0.48 | 3.99±0.43 | 19.03±2.10 | 7.73±0.83 | 6.83±0.73 | -10.60±1.52 | -11.05±1.49 | -0.45±0.91 |
| 2 | 43.65±1.50 | 52.00±0.77 | 52.70±0.99 | 9.94±1.06 | 5.11±0.54 | 6.57±0.70 | 22.79±2.54 | 9.82±1.05 | 12.46±1.35 | - 8.35±1.69 | - 9.05±1.80 | -0.70±1.25 |

explained anemia, and lobar pneumonia showed changes similar to those encountered in the second and third groups mentioned above.

*Degree of Precipitate and of Clumping of Erythrocytes.*—Table I indicates the amount of precipitate produced when blood serum was mixed with various diluents in 1:200 dilutions. This is the dilution commonly employed in making red blood cell counts. Also the degree of clumping of red blood corpuscles in the diluted blood of a patient with kala azar and in that of a normal person are compared. It is shown (Figs. 1 and 3) that the clumping is due, at least in large

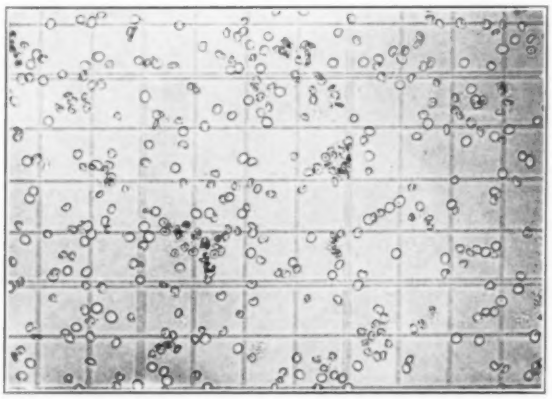

Fig. 6.—Photograph of counting chamber (×180) showing slight clumping of red corpuscles from the capillary blood of a normal person. Hayem's solution used as diluent.

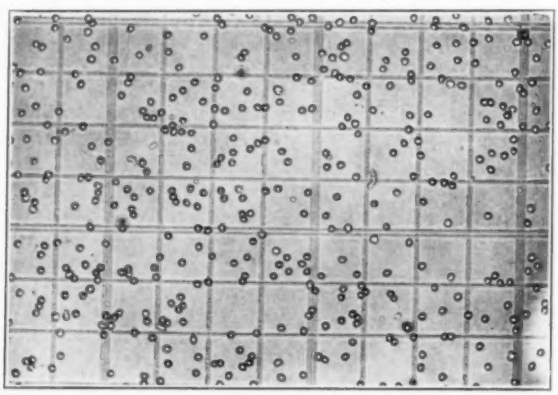

Fig. 7.—Photograph of counting chamber (×180) showing no clumping of red corpuscles from the capillary blood of a normal person. Gowers' solution used as diluent.

part, to collections of erythrocytes on coarse masses of coagulated material, presumably protein, in plasma or serum. Bichloride of mercury is responsible for the coarse precipitate in both Hayem's solution and Jorgensen's solution. Gowers' solution also produces a precipitate (Fig. 5) after standing for a time, but this precipitate is exceedingly fine and evenly divided and does not bring about clumping or other alterations in the distribution or appearance of the corpuscles (Figs. 4, 7).

Table II indicates the degree of clumping of corpuscles in a miscellaneous group of patients. This table shows the number of clumps in the whole ruled

area of the counting chamber. It is to be noted that no clumps were present when either isotonic solution of sodium chloride or Gowers' solution was used. The clumps were most numerous in the diluted blood of patients with kala azar, but were present also in large numbers in the diluted blood of patients with other diseases (Fig. 2 and Table II). The clumping was much more intense in the diluted blood of some patients than in others. The phenomenon was exhibited (not shown in the table) to a very much lesser degree in the blood of some apparently normal persons (Fig. 6), but in normal blood the process usually was not sufficiently marked to attract attention or to introduce significant error. The clumping was more pronounced when oxalated venous blood was used than when fresh capillary blood was employed. An attempt was made to correlate the degree of clumping with the values for total protein, albumin, globulin, and euglobulin in the blood serum of our patients. No correlation was apparent, but the data were inadequate to justify a final conclusion on this point.

The red blood cell counts on the blood of patients suffering from kala azar and nephritis in which Hayem's solution was used, exhibit on inspection lower values and on the whole much greater variation than those counts in which Gowers' solution or isotonic solution of sodium chloride was employed. This variation, however, is not easy to visualize and without critical study may be regarded as within the limits of technical error. In order to evaluate the data more accurately they were subjected to statistical analysis[17] as follows:

*Statistical Analysis of Nine Control Cases (Table IIIA).*—The means check well in each instance, except in Cases 4 and 5 in which the differences in the means with Hayem's solution and Gowers' solution are $6.20 \pm 1.56$ (Case 5) and $3.80 \pm 1.14$ (Case 4) and that with Hayem's solution and isotonic saline is $5.80 \pm 1.78$ (Case 5). These three values are greater than three times their probable errors and are considered to be significant. As to the standard deviation and the coefficient of variation, the values in each case are close. The averages of the means, standard deviations, and coefficients of variation all check well with these three solutions.

*Statistical Analysis of Sixteen Cases of Kala Azar (Table IIIB).*—It should be stated that not all of the cases of kala azar exhibited marked clumping of the erythrocytes. The first eleven cases listed exhibited these phenomena to a greater extent than the remaining five. The mean obtained from Hayem's solution in each case is smaller than those obtained from Gowers' solution and isotonic saline, except in Cases 14 and 15. The differences of means in the first eleven cases (values ranging from $15.70 \pm 1.87$ to $5.94 \pm 1.40$) are much larger than those in the last five cases. This is associated with marked clumping in the first eleven cases. The standard deviations and coefficients of variation calculated for Hayem's solution are much larger than those for the other solutions in each case, except those numbered 10 and 12. The average of the means with Hayem's solution ($55.65 \pm 1.75$) is much smaller than those with Gowers' solution ($62.51 \pm 1.49$) and isotonic saline ($62.47 \pm 1.57$). The averages of the standard deviations ($8.39 \pm 0.30$) and coefficients of variation ($15.54 \pm 0.69$) obtained for Hayem's solution are much larger than those obtained for Gowers' solution ($5.35 \pm 0.17$ and $8.67 \pm 0.30$) and isotonic saline ($4.81 \pm 0.10$ and $7.88 \pm 0.05$); while the cor-

responding average values from the studies with Gowers' solution and isotonic solution of sodium chloride check closely.

*Statistical Analysis of Two Cases of Nephritis (Table IIIB).*—The results of the analysis show findings comparable to those in the cases of kala azar.

## SUMMARY AND CONCLUSIONS

1. Hayem's solution, contrary to accepted belief, is unsatisfactory as a diluent for red blood corpuscle counts of some patients because it produces a coarse precipitate in the blood plasma and brings about clumping of erythrocytes, with resulting uneven distribution and significant error in the counts.

2. This unsatisfactory effect is marked in the blood of certain patients with kala azar, cirrhosis of the liver, pneumonia, nephritis, and other disorders. It is slight and of little significance in normal blood and in the blood of many patients with a variety of diseases.

3. The clumping of erythrocytes when blood is diluted with Hayem's solution may be thought, on superficial examination, to represent autoagglutination of the corpuscles. It appears that this error of interpretation has been recorded in the medical literature.

4. Statistical analysis of erythrocyte counts of a group of patients exhibiting the clumping of red blood cells has shown that the error with Hayem's solution is significantly great whereas the use of Gowers' solution introduces no appreciable error.

5. It is believed that in the hands of careful and well-trained workers there is no significant error to be encountered with the use of Hayem's solution in the counting of erythrocytes of normal individuals. However, on account of the presence of precipitated material, even in the blood of normal persons, there is introduced the factor of rapid sedimentation of the corpuscles in the pipette which favors the possibility of error in the hands of the less well-trained intern or technician.

6. Isotonic solution of sodium chloride may be used as a diluent but is open to serious objection because of its tendency to produce hemoylsis in the pipette and counting chamber, even when great care is used to prevent this complication.

7. Gowers' solution, made according to the formula presented by Mallory and Wright, possesses most of the essential properties of a diluent and is more satisfactory than any other diluting fluid described in the literature or tried in our experiments.

8. It would appear that the data from previous studies of the color index, mean corpuscular volume, and mean corpuscular hemoglobin of the blood of patients suffering from severe anemia, cirrhosis of the liver, nephritis, and some other diseases and in which Hayem's solution has been used for erythrocyte counts, may not be strictly correct in the light of the findings here reported.

We are indebted to Dr. I. C. Yuan and Mr. C. S. Hsueh of the Department of Public Health for their suggestions and help in the statistical analysis.

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# A MOUSE HOLDER FACILITATING INTRAVENOUS INOCULATIONS*

KENNETH L. BURDON, SC.M., PH.D., NEW ORLEANS, LA.

INTRAVENOUS injections of mice are rarely performed, despite the fact that inclusion of this route of inoculation would obviously enhance the value of many of the experimental studies for which these animals are now so widely used. Apparently, experimenters are generally unaware of the ease with which mice may be injected intravenously when a suitable holder is available.

The apparatus pictured here (Figs. 1 and 2) has proved highly satisfactory for this purpose in the writer's hands during more than two years of frequent use. It consists essentially of a housing for an electric light, the roof of which is slotted to permit illumination of the tail of the mouse from below, while the animal is held securely, but comfortably, in a simple wire cage. The whole contrivance is tilted so that the tail of the mouse is lower than its head, and gravity plus the heat from the electric bulb helps to dilate the tail veins, while at the same time these veins are made easily visible by the strong light from below. With the aid of this device experiments requiring repeated intravenous injections into the same mouse may be undertaken by a single worker without

*From the Department of Pathology and Bacteriology, Louisiana State University Medical Center.

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assistance and without fear of failure. Bleeding from the tail veins is also facilitated.

The structure of the apparatus is shown in the accompanying figures. The rectangular wooden box, open at the bottom, is of sufficient width to contain a 100-watt bulb and socket, which is fastened to one end, and of sufficient length to leave a space of about 9 cm. beyond the bulb at the opposite end (Fig. 1). Over this space is the slotted portion of the top (*A*). The end to which the socket is attached is elevated about 3.5 cm. above the table by a metal foot (*B*), in order to give the desired downward tilt to the cage. The box is lined with insulating material at sides and top, except over the slotted area. This prevents excessive heating of the exterior of the box, and, in the few minutes during which the light is on, only a comfortable warmth penetrates to the animal above.

The slot (*A*) is 0.5 cm. wide and 5.5 cm. long. Just beneath it is mounted a flat strip of metal (*C*) which extends out of the box in front where it may be

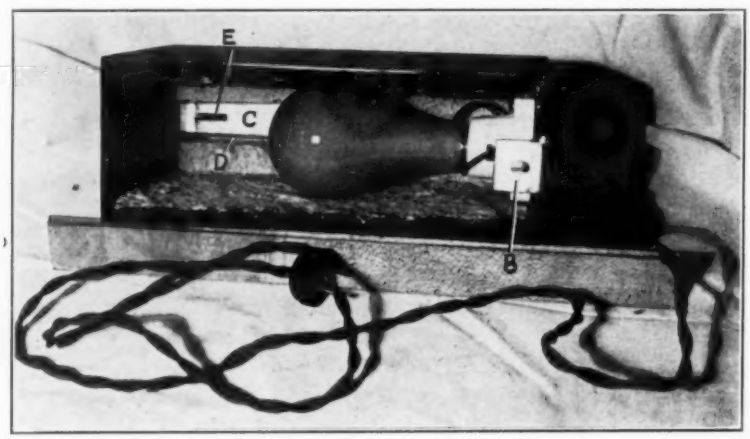


Fig. 1.

grasped by the fingers, and which slides straight forward or backward in metallic grooves (*D*) attached to the underside of the box top. This movable mask (*C*) has a single opening (*E*), 2.5 cm. long, parallel to the slot, and by adjustment of the position of this opening any desired portion of the tail of the mouse may be illuminated. Restricting the light in this way to a short space reduces glare and promotes accurate work.

The animal is held in a wire cage of roughly semicylindrical shape, about 10 cm. long and 3.5 cm. in diameter. This is placed just beyond the end of the slot (*A*), and hinged to the box top at the upper, open end (*F*). The cage is raised to admit the mouse, then lowered over the animal, and secured by the pivoting buttons (*G*), while one hand holds the protruding tail. The mouse is prevented from moving forward by placing in the open end of the cage a soft, cotton-stuffed cloth pillow (*H*) and pressing the pillow down against the animal as much as necessary. The end plate (*I*) is made purposely thick (3 mm. or more) so that the edges of the central opening through which the tail passes may be smoothly rounded to prevent trauma.

In making intravenous injections the tail is held near its tip by the fingers of the left hand and directly over or within the illuminated slot. The veins are clearly seen, and all that is necessary to bring them out sharply is to give the tail a few gentle strokes with a pledget of cotton moistened with alcohol. Needles of 26 or 27 gauge are used. It is best to grasp the barrel of the syringe firmly between the index and middle fingers of the right hand, holding the thumb over the plunger. After a little experience one can easily tell when the vein has been entered, and the slightest pressure on the plunger will answer the question, for if the needle is indeed within the vein the inoculum will pass in rapidly and without resistance.

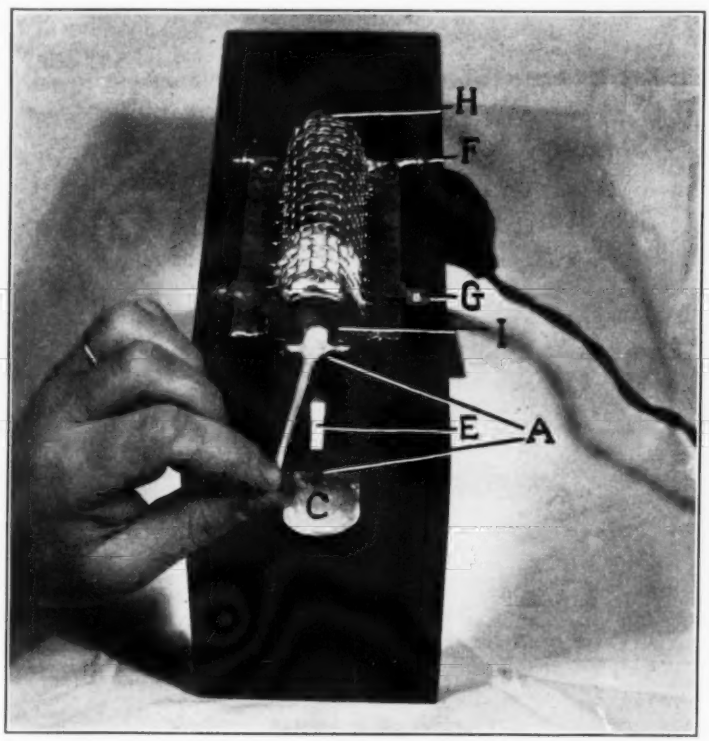


Fig. 2.

For bleeding, a puncture may be made directly into a clearly visible vein, and by alternately stroking the tail downward above the puncture and compressing it below the puncture, drops of blood will appear which may be collected in a capillary pipette.

## SUMMARY

An apparatus has been described which facilitates the making of intravenous injections into the tail veins of mice. Bleeding from these veins is also made easier. The device makes possible the utilization at will of an important technique by the individual worker, and greatly enlarges the potential usefulness of one of our most serviceable laboratory animals.

# A NOTE ON THE PREPARATION OF THE UREASE REAGENT FOR WRENN'S METHOD FOR THE DETERMINATION OF UREA IN BLOOD AND URINE*

DAVID B. SABINE, B.S., YONKERS, N. Y.

WRENN'S method[1] is admirably adapted for the determination of urea nitrogen in blood and urine by direct nesslerization, but the preparation of the urease reagent is long and complicated. If Arleo Urease be used in place of Arleo Jack Bean Meal, the reagent can be prepared ready for use in a few minutes, instead of a couple of days.

To make a solution of approximately the same strength as Wrenn's, dissolve 2 gm. of Arleo Urease in 10 c.c. of water. (This solution is not clear, but its opacity will not in any way affect its usefulness. Although it may be centrifuged clear without any immediate diminution in ureolytic power, it will not be as stable, probably because of the loss of the protective colloids.) To this solution add an equal volume of C.P. neutral glycerin. When standardized by Wrenn's method,[1] this solution will yield 50 to 100 mg. of nitrogen from the Summer urea phosphate substrate.

A solution so prepared and kept well stoppered in the refrigerator has retained its activity undiminished for four months. (A solution which was centrifuged clear before the addition of the glycerin, lost approximately half of its activity under the same conditions.)

Inasmuch as this solution has the same ureolytic power and the same stability, it can replace the solution prepared by the more complicated method described by Wrenn.

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*From the Laboratories of the Arlington Chemical Co., Yonkers.
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# AN IMPROVED TOURNIQUET

JOHN R. WILLIAMS, M.D., ROCHESTER, N. Y.

IT IS common practice in physicians' offices and clinics to use round rubber tubing, 1 cm. or less in diameter, for tourniquets. This tubing is of varying degrees of elasticity, and when applied to a patient's arm under tension it may cause considerable discomfort. For some time the author has used a flat thin-walled tubing which has many advantages over the round type. It resembles the uninflated inner tube of a tire, and may be obtained in various sizes and

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widths. Commercially it is used in the making of rubber bands. The width best suited for clinical purposes is 2 cm.

Some of the advantages of the flat tubing over the round are:

1. It does not slip nearly so readily when applied to the arm.
2. It does not pinch or cut the flesh, or cause other discomfort.
3. When applied properly, because of its increased width, it interrupts only the venous flow; thus the veins stand out more conspicuously than they do when the arterial flow is also occluded, as may occur with the application of the round type.
4. It is very inexpensive and may be obtained through a dealer in rubber goods. It costs about ten cents an ounce, and one ounce is ample for a good tourniquet.

388 Monroe Ave.

## LANTERN SLIDES OF PHOTOMICROGRAPHS IN COLOR

### A Simplified Method

W. Ray Jones, M.D., Seattle, Wash.

THE principle is that of changing the silver on an ordinary lantern slide of a photomicrographic field so that it will fix dyes corresponding to the original colors. Aniline colors set thus are exceptionally resistant to heating in the projector without browning.

#### STEPS

1. Print the slide lighter than it is to be when finished, fix, and wash as usual. While still wet immerse in the mordanting solution, keeping in constant agitation until a chocolate brown color first appears upon the half tones. This requires two to twenty minutes, depending upon strength and freshness of the solution.
2. Agitate in cold running water one or two minutes. Insufficient washing leaves a yellowish tinge and causes precipitation of a dye sludge all over the film. Overwashing prevents the dye from taking hold.
3. Agitate in the dye for from one to fifteen minutes, until the proper amount of color has been deposited. During the dyeing rinse in fresh water frequently and use a cotton pledget to wipe the dye sludge off the surface.
4. Wash for half an hour, dry, and mount.

#### VARIATIONS

1. A two color combination as hematoxylin and eosin can be obtained by dyeing with gentian violet and then using as a cover glass mounting, a clear gelatin slide lightly stained with safranine or eosin.

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2. True two colors without overlapping can be secured by using the one color finished slide and printing another very thin slide in reverse. Dye this second slide the lighter color (red) and mount the two properly superimposed upon each other. Clear areas in the field on the second slide which are supposed to remain as the lumen of a duct, need be either hand painted out on the first before making the second printing (opaque on the glass side washes off without injuring the film), or after printing be reduced, using Farmer's solution and extra ferricyanide on a cotton pledget. Different shades and other colors can be secured, as by staining yellow first and then red to secure a green. Blood pictures can be tinted by staining light blue first and then red to obtain a Wright's stain color.

SOLUTIONS

| MORDANT STOCK | AVOIRDUPOIS | METRIC |
|---|---|---|
| Uranium nitrate | 15 gr. | 1.0 gm. |
| Oxalic acid | 7½ gr. | 0.5 gm. |
| Potassium ferricyanide | 7½ gr. | 0.5 gm. |
| Water made up to | 4 oz. | 120.0 c.c. |

Dissolve ingredients separately and mix in order given. For use, dilute with four parts of water. This stock keeps poorly, being decomposed by either light or heat.

| DYE | AVOIRDUPOIS | METRIC |
|---|---|---|
| For coloring use either: | | |
| Gentian violet | 1½ gr. | 0.1 gm. |
| Methylene blue | 3 gr. | 0.2 gm. |
| Safranine (red) | 6 gr. | 0.4 gm. |
| Auramine (yellow) | 6 gr. | 0.4 gm. |

Dissolve the dye in an ounce of hot water, filter, and add:

| | | |
|---|---|---|
| Acetic acid 10 per cent | 1-3 oz. | 10 c.c. |
| Water up to | 8 oz. | 500 c.c. |

TECHNICAL AIDS

1. Be exact. Color once in cannot be removed. If insufficient color sets, one can remordant and stain again.

2. Any iron exposed in the trays may ruin the mordant.

3. Hypo must all be washed away before mordanting.

4. Slides left in close contact for any time during any of the processes stain unevenly.

5. Train the eye to judge when sufficiently mordanted and when coloring is right.

6. As solutions weaken, times required, lengthen. They can be reinforced by adding fresh stock. Five ounces of the diluted mordant if freshly made and the eight ounces of dye should care for from six to eighteen slides.

Weakened acidulated chlorox (an alkaline solution of hypochlorite), removes these dyes from the fingers, pans, etc., but ruins the slide.

The slidemaker need himself be a microscopist to duplicate the colors properly. An amateur photographer can make excellent prints on glass easier than on paper. The book *How to Make Good Pictures*, Eastman Kodak Co., 50c, and other beginners' books will assist the novice in mastering the technique.

# SIMPLIFIED METHOD FOR MAKING PHOTOGRAPHIC RECORDS OF PETRI DISH CULTURES WITHOUT CAMERA*

EUGENE MAIER, PH.D., VENICE, FLA.

IN LABORATORIES where it is desirable to keep permanent records of Petri dish cultures a considerable saving of time may be effected by making direct prints on sensitized paper; this method is particularly valuable where camera equipment is not easily available. All that is required are sensitized paper, an electric light bulb, and the ordinary facilities for developing and fixing photographic prints.

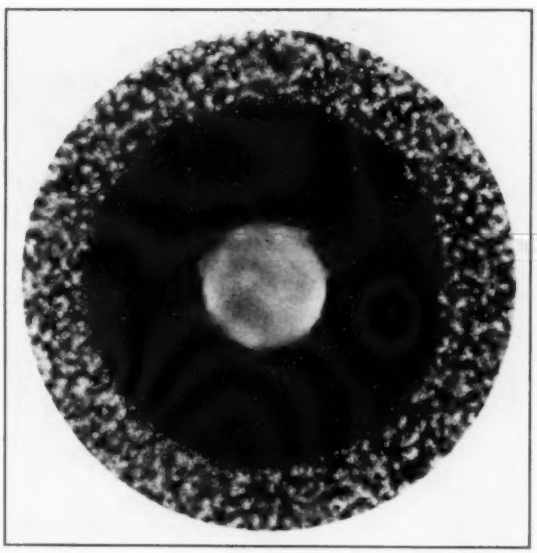

Fig. 1.

For the purpose of demonstrating the value of this method, we have found the agar cup technique of Himebaugh[1] the best. A Petri dish is prepared with clear nutrient agar in the ordinary manner and inoculated with the organism to be tested; a circle of about 15 mm. diameter is cut in the center with the open end of a sterile test tube and the material inside the circle removed, leaving a circular hole, or cup, in the center. Into this space is inserted the preparation to be used in the test. The Petri dish is then placed in the incubator for twenty-four hours, after which time sufficient growth will have taken place for the print to be made. It is then removed to a dark room and placed over a piece of sensitized paper,† of suitable size and shape, about 1.5 to 2.0 meters from an electric light bulb of 75 or 100 watts, the Petri dish being between the paper and the light. Exposure is made for about ten to twenty-five seconds, but both time of exposure and distance can only be determined by trial. The print is then developed and fixed by the usual methods.

*From the Florida Medical Center, Venice.
Received for publication, January 17, 1938.

†In our experience the best results are obtained with Azo paper, made by the Eastman Kodak Co.

In the illustration shown, the agar plate was made by inoculation of organisms taken from the mouth, and prepared according to the technique of Heimbaugh, described above. The light disk in the center is the area of the germicide, the dark zone is that of inhibition where no visible growth took place, and the mottled ring on the outside shows the growth of bacteria. The sharp line of division between the dark zone and the outer ring is a peculiarity of the material used in this particular instance.

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## A METHOD FOR THE PROTECTION OF MICROSCOPIC MOUNTS FOR MAILING*

HARVEY P. BARRET, M.D., AND THERESA CULP, A.B., CHARLOTTE, N. C.

A NUMBER of uses have been found for the material parafilm.† Of special interest to the writers have been the various applications of this product to laboratory procedures. It has been used very efficiently by Terry[1] in the sealing of culture tubes, Petri dishes, and other containers, its chief advantage being the retention of moisture or prevention of drying out of cultures. Another advantage not mentioned, especially in culture vessels, is the exclusion of contaminating air. Its superiority over cotton plugs alone or cotton plugs with a paraffin coating has apparently been clearly demonstrated. There are many grades of parafilm for different purposes. That most suitable for laboratory use is designated as Parafilm Grade M.

All laboratory workers know of the many difficulties and inconveniences in the shipment, by mail or otherwise, of prepared slides; that is, slides on which a cover glass has been placed using Canada balsam or other sealing medium. The stickiness of the balsam, the adherence of the cover glass and slide to the sides of the container, and the slipping of the cover glass over the mounted object are some of the difficulties encountered. The writers have found that a strip of parafilm wide enough to cover both the cover glass and a portion of the slide at each end of the cover glass, if placed over the entire upper surface of the slide and carried under a few millimeters on each side, will protect the mount and hold it very securely in place. For use with an ordinary 3 by 1 inch slide and a 40 by 22 mm. cover glass, the parafilm is cut in strips 2¼ inches wide by 1¾ inches long. This allows space on the end of the slide for labelling. In using other sizes of cover glasses, the strips of parafilm may be cut accordingly. One edge of the parafilm is fastened along its entire length to the underneath surface of the slide by gentle rubbing with the fingers. This is especially necessary if the

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†The parafilm may be obtained from the Menasha Products Co., Menasha, Wis.

slide is cold. We have found that by slightly warming the parafilm it will adhere to the slide with a minimum of pressure or rubbing. In practice, while preparing the slides for shipment, the authors place the cut strips of parafilm on the laboratory table beneath or in the vicinity of the microscope lamp. This small amount of heating is sufficient for the proper softening of the parafilm. The parafilm is then drawn tight and brought up over the mounted section of the slide and attached in the same way to the undersurface of the slide on its opposite side. The portion of the parafilm which overlaps the ends of the cover glass is then rubbed gently in order to attach the parafilm to the uncovered upper surface of the slide. If desired, the parafilm covering the cover glass may be rubbed gently in order to attach it to the upper surface of the cover glass. As our experience has been with freshly made preparations, we have refrained from exerting pressure on the cover glass. Using this method, we have been able to mail numerous slides that have been freshly prepared. In fact, this is the chief advantage of the method. There is no need for the delay caused by waiting for the drying of the balsam. Our routine method has been to prepare the slides, to wrap them immediately in parafilm, to place a piece of waxed paper or cardboard between the slides, and then to put the slides in an ordinary mailing case without any further preparation. A small mailing case, 1¾ inches inside diameter, will easily hold twenty slides after they have been wrapped in parafilm and waxed paper. It is not advisable to place a rubber band or any other constricting material around the package of slides because of the pressure exerted on the fresh balsam under the cover glasses. The parafilm is easily unwound from the slide. If any particles of parafilm are left on the slide, they are easily and quickly removed with a cloth moistened with xylol. We have used this method on several hundred slides and have met with none of the inconveniences and casualties encountered previously in mailing fresh slides.

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612 Professional Bldg.

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# AN IMPROVED METHOD OF EXAMINATION OF SPINAL FLUID FOR THE DIAGNOSIS OF GENERAL PARESIS*

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*Reading of the Colloidal Gold Curve After Forty-Eight Hours.*—The colloidal gold curve is usually read after twenty-four hours. This time is sufficient for the diagnosis when the results are unequivocally positive or negative. However, in incipient cases in the very first stage, in transitional cases, and after intensive treatment, one is often in doubt as to what conclusion to draw

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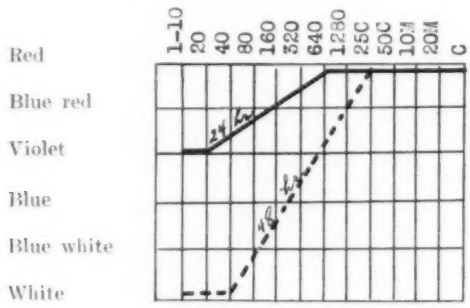
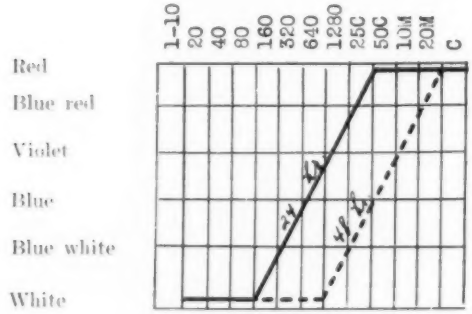
from the reading. In these cases the herewith suggested delayed reading of the gold curve helps to surmount the difficulties. Normal fluids show hardly any change after twenty-four hours. Instead of many explanations, several examples will help to clarify this point.

Chart 1.




Clinical: G.P. incipient
Wass. R.: 0.05 +++
Hemolysin: Negative
Protein: +++
Cells: 60

Chart 2.




Treated G.P.
Wass. R.: 0.2 +++
Hemolysin: Negative
Protein: Positive
Cells: 32

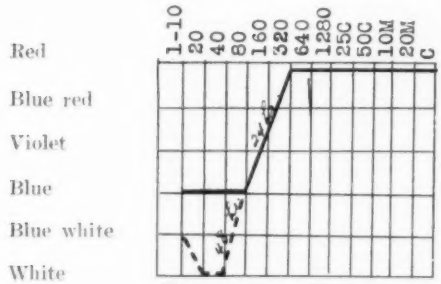
The high concentrations from 0.2 to 0.5 c.c. are all from treated cases whose gold curve is still typical, whose content of Wassermann reaction antibodies is already diminished. Without the above suggested evaluation with decreasing amounts, a differentiation of the insufficiently treated or untreated cases from the well-treated cases would often be impossible. Even if we are at a loss as to the case history, the Wassermann reaction evaluated with decreasing amounts permits conclusions concerning the efficiency of the applied therapy and suggests proper directions for subsequent treatment.

As one can see from these examples, doubtful colloidal gold curves become absolutely unequivocal after forty-eight hours. This is true chiefly in cases of types 1 and 4, which presented the clinical picture of incipient paresis, but which also permitted different interpretations. The delayed reading clearly decided for general paresis, which was confirmed by the subsequent course.

On the other hand, clear-cut cases of tabes dorsalis (Chart 3) never show a typical paretic curve after forty-eight hours. On the contrary, in spite of the development at first of a doubtful gold curve after twenty-four hours, a typical tabetic curve develops on further standing.

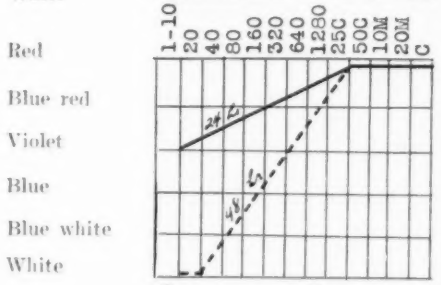
This observation can also be utilized for the standardization of the colloidal gold solution. Even if the same procedure in the preparation of the gold solution is always followed, the solutions prepared at different times are not always the same in their behavior. The standardization must be done at first with salt

Chart 3.



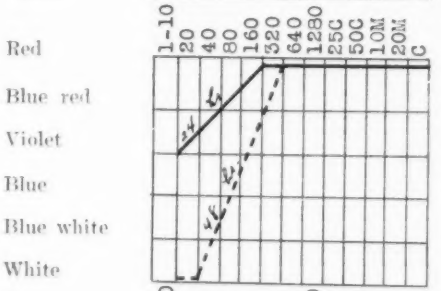

Clinical: Tabes dorsalis
Wass. R.: 0.1 +++
Hemolysin: Negative
Protein: Positive

Chart 4.



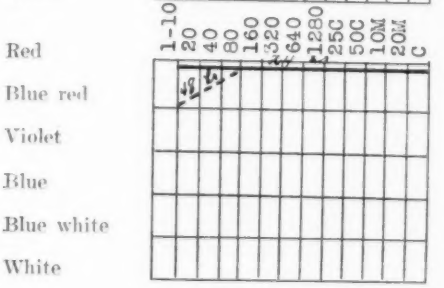

Early G.P.
Wass. R.: 0.3 +++
Hemolysin: Negative
Protein: ++
Cells: 200

Chart 5.




Taboparesis
Wass. R.: 1.0 +++
Hemolysin: Negative
Protein: Trace
Cells: 0

Chart 6.




Latent syphilis, infection 1913
Wass. R.: 1.0 +
Protein: Negative
Hemolysin: Negative
Cells: 2

Eighty-three paretic fluids gave completely positive reactions in the following dilutions:

| | | | |
|---|---|---|---|
| 0.5 | c.c. | 5 fluids | |
| 0.4 | c.c. | 1 fluid | |
| 0.3 | c.c. | 11 fluids | |
| 0.2 | c.c. | 8 fluids | |
| 0.1 | c.c. | 15 fluids | = 18 per cent |
| 0.05 | c.c. | 28 fluids | = 34 per cent |
| 0.025 | c.c. | 11 fluids | = 11 per cent |
| 0.012 | c.c. | 1 fluid | |
| 0.006 | c.c. | 3 fluids | |
| | | 83 fluids | |

= 65 per cent

solutions of various concentrations (from 0.2 to 0.9 per cent). With the concentration of the salt solution thus found, the clinical usefulness has to be tested on normal fluids and those of paresis. Only those gold solutions prove absolutely dependable which also show typical curves after forty-eight hours.

*Evaluation of the Fluid With Decreasing Amounts.*—The ordinarily used doses from 1 c.c. down to 0.2 c.c. are too high for general paresis; they afford no possibility of differentiation. If, however, one examines amounts of fluid down to 0.003 c.c. according to the technique suggested by me,[1] one can often make the diagnosis of general paresis from the Wassermann reaction. No other form of the disease gives such sensitive reactions.

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# A MICROCHEMICAL METHOD FOR THE DETERMINATION OF MANGANESE BY OXIDATION WITH POTASSIUM PERSULFATE*

## A Critical Study of the Persulfate Method

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THE wide distribution of manganese in biological material, pointing to the metabolic importance of this element, has created wide interest in its study. This fact makes it highly desirable to have a dependable method for the quantitative detection of small amounts of manganese in such material. These determinations are usually made by the colorimetric method, the manganese being converted into the manganous salt which is then oxidized to permanganic acid. The resulting color is compared with a standard manganous salt similarly treated. The oxidation may be accomplished in a number of ways. Lead dioxide[1] was the first oxidizing agent used. Sodium bismuthate,[2] potassium periodate,[3] and ammonium or potassium persulfate employing silver nitrate as a catalyst[4] are also used for this purpose. Although the latter means of oxidation has been extensively employed, it is known that the method frequently fails to produce the true permanganic acid color, oxidation may not be complete, and interfering colors often appear. Wester,[5] working with nitric acid, and Newcomb and Sankaran,[6] using a mixture of nitric and phosphoric acids, have tried to overcome these difficulties, but they have done little more than to make the adjustments necessary for their own specific problems. Inability to control the persulfate method has led to its replacement by the periodate method of Willard and Greathouse[3] which is said to be free from such objections. The persulfate method, however, has distinct advantages over other means of oxidation. Persulfate is cheaper than periodate, and when solutions are colored brown due to carbon, persulfate must be used to remove the color even if the periodate method is employed.

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The belief that the unsatisfactory aspects of the persulfate method could be avoided, and that it could be developed into a dependable one has led to this investigation, and has resulted in the observation that failure to adjust the acid to the amount of catalyst used has been the whole cause of the trouble. The findings are recorded in graph form, showing the relative amounts of acid and catalyst required for optimum performance.

*Reactions Involved.*—According to Marshall[4] the reactions are as follows:

$$4(NH_4)_2S_2O_8 + 3H_2O = 7NH_4HSO_4 + HNO_3 + H_2SO_4.$$

The catalyst is thought to be silver peroxide:

$$Ag_2S_2O_8 + 2H_2O = 2H_2SO_4 + Ag_2O_2.$$

Travers,[7] however, believes the catalyst is silver peroxynitrate:

$$7AgNO_3 + 3(NH_4)_2S_2O_8 + 6H_2O =$$
$$3Ag_2O_2.AgNO_3 + 3(NH_4)_2SO_4 + 3H_2SO_4 + 6HNO_3.$$

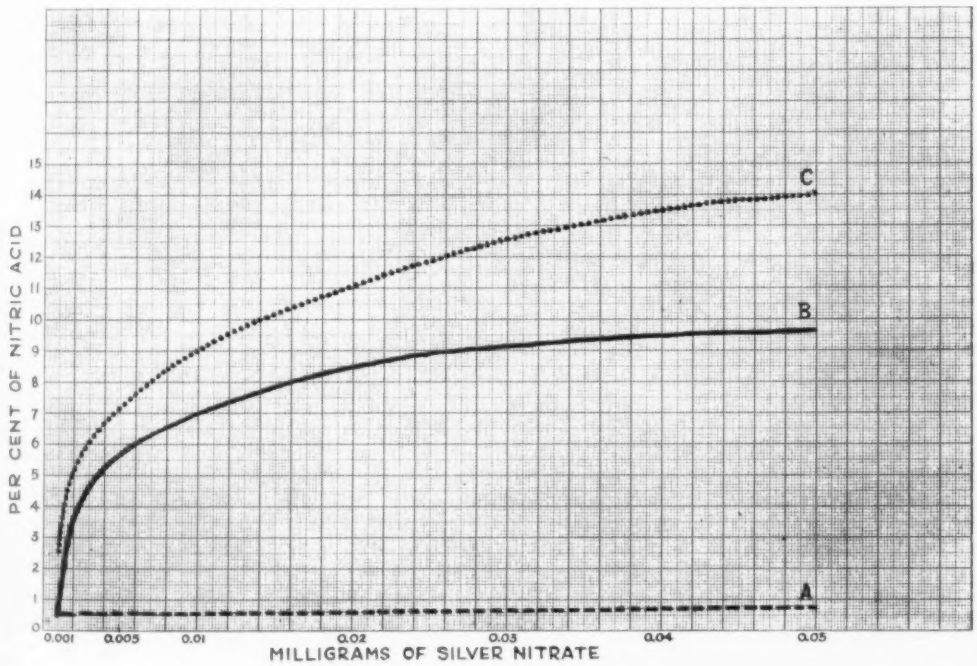


Chart 1.

The oxidation of manganese by persulfate can be brought about in the presence of nitric acid, sulfuric acid, phosphoric acid, or in a mixture of these acids. The present work was done with nitric acid alone. Further study is in progress in which sulfuric acid and phosphoric acid are used, because the conditions for oxidation are different with different acids.

## OBSERVATIONS

Much has been said about the proper degree of acidity of the solution, but the question of adjusting the per cent of acid to the particular amount of silver nitrate used to catalyze a reaction has heretofore been overlooked. Inspection of the accompanying charts, however, shows that the amount of catalyst employed is the factor which determines the per cent of acid required,

and without giving first consideration to this matter the amount of acid that must be added is left to chance.

The literature describing this means of oxidation is vague and conflicting, requiring each person using the method to find by trial and error the conditions best suited to his work. Some observers make the mistake of developing the color in a small volume and then diluting it to a larger one. This procedure is permissible only if all the reacting constituents are correctly adjusted for developing the color in the small volume and then readjusting the acid when the dilution is made. Chlorides are sometimes removed from the solution by adding silver nitrate, the silver chloride filtered off, and the remaining silver nitrate used to catalyze the reaction. Here, since the amount of silver

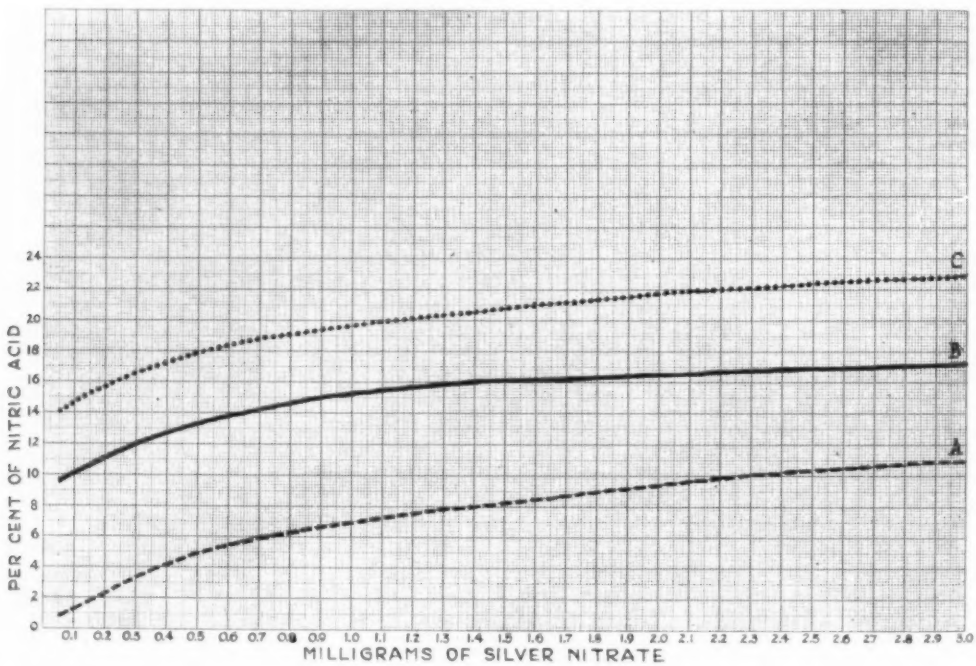


Chart 2.

nitrate left in the solution is not known, there is no way of telling how much acid to use.

The accompanying charts giving silver nitrate in milligrams at the abscissa and acid in per cent at the ordinate, show by inspection the relative proportions of catalyst and acid to use with manganese, ranging in amounts from less than 0.001 mg. to 0.5 mg. Two sets of graphs are necessary due to the great range of conditions covered. The proportions of catalyst and acid indicated in Chart 1 are correct for amounts of manganese up to about 0.06 mg., but for greater quantities Chart 2 should be consulted. With any given quantity of silver nitrate used to catalyze a reaction, the allowable range of acid is indicated by curves A and B, curve A showing the minimum and curve B the maximum amount. The positions on the curves defining these limits are directly above the point designating the amount of silver nitrate being

employed. If less acid than that shown by curve A is used, manganese or silver, or both, will appear in colloid form, imparting a red or brown color to the solution. Use of more acid than that indicated by curve B will result in the development of a pure permanganic acid color but of less intensity than the maximum. This depth of color will decrease as the acid increases, and will continue to do so until that amount of acid shown in curve C is exceeded where no color at all will appear.

TABLE I

RESULTS OF DUPLICATE ANALYSES AND RECOVERY OF ADDED MANGANESE

| SUBSTANCE | WT. OF SUBSTANCE | MN ADDED | MN FOUND | RECOVERY OF ADDED MN |
|---|---|---|---|---|
| | *gm.* | *mg.* | *mg.* | *per cent* |
| Human liver | 6.0 | | 0.0118 | |
| Human liver | 6.0 | | 0.0112 | |
| Human liver | 6.0 | 0.0025 | 0.0141 | 104.0 |
| Beef liver | 2.00 | | 0.0054 | |
| Beef liver | 2.00 | | 0.0057 | |
| Beef liver | 2.00 | 0.0035 | 0.0088 | 94.3 |
| Beef liver | 1.20 | | 0.0032 | |
| Beef liver | 1.20 | | 0.0035 | |
| Beef liver | 1.20 | 0.0065 | 0.0094 | 92.3 |
| Rabbit liver | 2.00 | | 0.0052 | |
| Rabbit liver | 2.00 | | 0.0058 | |
| Rabbit liver | 2.00 | 0.0035 | 0.0089 | 97.1 |
| Beef muscle | 12.00 | | 0.0048 | |
| Beef muscle | 12.00 | | 0.0040 | |
| Beef muscle | 12.00 | 0.0055 | 0.0097 | 96.4 |
| Human gallstone | 1.25 | | 0.0046 | |
| Human gallstone | 1.25 | | 0.0044 | |
| Human gallstone | 1.25 | 0.0065 | 0.0112 | 103.1 |
| Human gallstone | 1.00 | | 0.0038 | |
| Human gallstone | 1.00 | | 0.0040 | |
| Human gallstone | 1.00 | 0.0065 | 0.0108 | 106.1 |
| Lettuce | 0.215 | | 0.0054 | |
| Lettuce | 0.215 | | 0.0055 | |
| Lettuce | 0.215 | 0.0035 | 0.0091 | 102.8 |
| Lettuce | 0.125 | | 0.0034 | |
| Lettuce | 0.125 | | 0.0034 | |
| Lettuce | 0.125 | 0.0055 | 0.0088 | 98.2 |
| Soy beans | 0.15 | | 0.0047 | |
| Soy beans | 0.15 | | 0.0045 | |
| Soy beans | 0.15 | 0.0045 | 0.0090 | 97.7 |
| Filter paper | 2.50 | | 0.0044 | |
| Filter paper | 2.50 | | 0.0041 | |
| Filter paper | 2.50 | 0.0045 | 0.0086 | 97.8 |

To catalyze a given amount of manganese, a considerable variation in silver nitrate is permissible, but insufficient catalyst results in incomplete oxidation of manganese. An excess of persulfate does not interfere with oxidation, and volume is important only in that it is convenient to develop colors at once of intensities suitable for comparison, thus avoiding the necessity of further dilution. With quantities of manganese up to 0.01 mg., 10 c.c. volumes are suitable, but as the amount of manganese becomes greater, the volume should be increased to produce the desired depth of color, consideration being given always to the per cent of acid used rather than to the total amount. With acid and catalyst used in the proportions suggested by the graphs, complete oxidation of manganese takes place, resulting in a pure permanganic acid color which is stable and of the maximum intensity.

The applicability of the procedure is illustrated by the results in Table I, giving the analyses of duplicate samples of a variety of biological materials and of filter paper, also showing the recovery of added manganese. Weighed amounts of liver and muscle were dissolved in nitric and sulfuric acids and aliquot parts used, while the other substances analyzed were dried to 105° C. Manganese for recovery was added before the material was prepared for oxidation.

TABLE II

RESULTS OF DUPLICATE ANALYSES USING DIFFERENT AMOUNTS OF CATALYST AND DIFFERENT PER CENTS

| SUBSTANCE | WT. OF SUBSTANCE ANALYZED | WT. OF $AgNO_3$ | $HNO_3$ | POTASSIUM PERSULFATE | VOLUME | MANGANESE FOUND |
|---|---|---|---|---|---|---|
| | *gm.* | *mg.* | *per cent* | *gm.* | *c.c.* | *mg.* |
| Human liver | 4.0 | 0.8 | 8 | 0.5 | 10 | 0.0072 |
| Human liver | 4.0 | 2.5 | 15 | 0.5 | 10 | 0.0075 |
| Beef liver | 1.85 | 0.5 | 8 | 0.5 | 10 | 0.0048 |
| Beef liver | 1.85 | 2.5 | 14 | 0.5 | 10 | 0.0045 |
| Rabbit liver | 2.00 | 1.0 | 8 | 0.5 | 10 | 0.0045 |
| Rabbit liver | 2.00 | 2.0 | 14 | 0.5 | 10 | 0.0050 |
| Beef muscle | 10.0 | 0.5 | 7 | 0.5 | 10 | 0.0032 |
| Beef muscle | 10.0 | 2.0 | 15 | 0.5 | 10 | 0.0038 |
| Human gallstones | 2.0 | 0.8 | 8 | 0.5 | 10 | 0.0071 |
| Human gallstones | 2.0 | 2.5 | 15 | 0.5 | 10 | 0.0073 |
| Lettuce | 0.2 | 0.5 | 6 | 0.5 | 10 | 0.0065 |
| Lettuce | 0.2 | 2.5 | 13 | 0.5 | 10 | 0.0063 |
| Soy beans | 0.6 | 0.4 | 6 | 0.5 | 10 | 0.0064 |
| Soy beans | 0.6 | 2.0 | 14 | 0.5 | 10 | 0.0068 |
| Filter paper | 6.0 | 0.5 | 7 | 0.5 | 10 | 0.0066 |
| Filter paper | 6.0 | 2.5 | 14 | 0.5 | 10 | 0.0062 |

The data recorded in Table II show the futility of trying to determine the amount of acid to be used without giving first consideration to the quantity of silver nitrate employed. Identical results are obtained on duplicate samples by using widely varying amounts of acid and catalyst. Inspection of the graphs, however, shows that in every instance, the per cent of acid used falls within the limits permitted for the amount of silver nitrate employed.

Organic matter was prepared for oxidation by wet combustion and by dry ashing. Amounts of material, sufficient to yield from 0.004 mg. to 0.01 mg. manganese, were digested in 50 c.c. round bottom pyrex flasks with nitric acid and sulfuric acid. When digestion was completed all except about two or three drops of the sulfuric acid were driven off, thus removing all chlorides and leaving the manganese in easy form for oxidation.

The ashing was done in a silica pipe[1] made for forcing air through the burning material (Fig. 1). Bradley,[8] who used clay pipes for this purpose, found the time of operation much shorter than when the ashing is done in crucibles.

*Oxidation.*—The digested material was oxidized in the digestion flask by adding about 8 or 9 c.c. of water, enough nitric acid to make 8 to 10 per cent on the basis of a 10 c.c. volume, 0.2 c.c. of a 0.25 per cent silver nitrate solution, and 0.5 gm. of potassium persulfate. The flask was put into a beaker of boiling water where the color develops in a few minutes. Digestion and oxidation in the same flask is desirable because then there is no loss from

spattering or from transferring of material, and no manganese escapes oxidation. Since nothing is added but nitric and sulfuric acids, and since these can be obtained manganese free, there is no danger of inadvertently adding the element. After the color is developed, the solution is transferred to a graduated centrifuge tube, and the volume adjusted to 10 c.c., including washings from the flask.

Sometimes a considerable amount of silica is left in the ashed material, which Newcomb and Sankaran[6] say will adsorb manganese. These workers suggest a means of removing manganese from ash containing much silica, but the procedure is open to the danger of adding the element with the chemicals used for fusion.

*Controls.*—In testing for minute quantities of manganese it is necessary to make many control determinations to be sure that the element is not being added as a contaminant. The Feigl test,[9] employed and described in detail by Ray,[10] shows that materials, including filter paper, which are supposed to

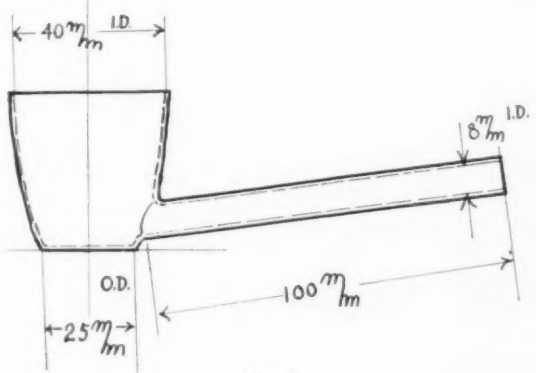


Fig. 1.

be chemically pure, often contain varying amounts of manganese. This fact emphasizes the advantage of preparing material for oxidation by wet combustion.

*Colorimeter Tubes.*—The color comparisons were made in Nessler tubes with polished bottoms, of uniform bore, of 10 c.c. capacity, graduated in tenths of a cubic centimeter, and 12 cm. in length. Such tubes are convenient to use, and permit accurate determinations on smaller amounts of manganese than with a colorimeter.

## CONCLUSIONS

A critical study was made to determine the conditions necessary for the complete oxidation of a manganous salt to permanganic acid in nitric acid using silver nitrate as a catalyst. The findings, showing the necessity of adjusting the acid to the particular amount of catalyst used, are recorded in graph form making it possible to determine, by inspection, the relative amounts of these constituents to use for optimum performance of the method. The experimental work covers a sufficiently wide range of conditions to meet the needs of most workers.

The silica pipe was made special for us by Arthur H. Thomas Co., Philadelphia.

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## THE KAHN REACTIONS OF SIXTY-FOUR TULAREMIA PATIENTS*

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BETWEEN November, 1936, and October, 1937, we received 87 sera from 64 tularemia patients. Clinical findings corroborated the positive agglutination reactions for tularemia encountered. Sera tested represented various stages in the disease, twelve sera having been submitted too early for the positive agglutination reaction to have developed. The Kahn test on these sera gave the following reactions:

KAHN REACTIONS (87 TESTS) ON SERUM FROM 64 TULAREMIA PATIENTS

| | | |
|---|---|---|
| Negative | Number of specimens | 78 |
| Doubtful | Number of specimens | 5 |
| | Clinical syphilis | 1 |
| | No syphilis | 1 |
| | Unknown diagnosis | 3 |
| Positive | Number of specimens | 4 |
| | Clinical syphilis | 4 |
| | No syphilis | 0 |

The patient with the false doubtful reaction had no clinical evidence of syphilis and a negative Kahn was obtained the same week.

SUMMARY

One tularemia patient of a group of 64 gave a false doubtful Kahn reaction. The specificity of the Kahn reaction is evidently not influenced in tularemic infections.

*From the Laboratory Section, St. Louis Health Division.
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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**CYANIDE, Toxicology of, Gettler, A. O., and Baine, J. O.** Am. J. M. Sc. **195**: 182, 1938.

As a preliminary rapid and sensitive way of ruling out the presence of cyanide the authors recommend:

1. *Copper-Guaiac Test.*—Place about 50 gm. of finely ground organ or a few centimeters of stomach contents into a 100 c.c. flask. Acidify the material with tartaric acid, and suspend above it a strip of freshly prepared copper sulfate-guaiac paper (filter paper which has been wet in turn with 10 per cent alcoholic solution of guaiac and 0.1 per cent aqueous copper sulfate solution), stopper the flask, warm contents a little and allow to stand about one-half hour. If a blue or green color does not develop on the paper, then cyanide is definitely excluded. No further tests for cyanide need be done, because this is the most sensitive test available. If a blue color develops, cyanide may be present. Since, however, the blue color is also produced by ammonia gas, hydrogen chloride, ozone, oxides of nitrogen, bromine, chlorine and hydrogen peroxide, two or three of the following tests must be resorted to in order to establish definitely the presence of cyanide in the material.

To establish the presence of cyanide they recommend:

2. *Liebig's Thiocyanate Test.*—Twenty cubic centimeters of the distillate and 2 c.c. of ammonium polysulfide are placed into an evaporating dish, and evaporated to dryness on the steam bath. The residue is treated with 5 c.c. of water, then slightly acidified with hydrochloric acid, warmed a little, thoroughly stirred and allowed to stand several hours, in order that the colloidal sulfur may precipitate. The material is then filtered, and 10 per cent ferric chloride is added dropwise, until a maximum color intensity is obtained. A red color indicates the presence of cyanide.

A variation of the thiocyanate test follows: An aliquot portion of the distillate is boiled with ammonium sulfide for a few minutes, then concentrated to 1 c.c., acidified with hydrochloric acid, and extracted several times with ether. The ethereal extracts are allowed to evaporate spontaneously. Dilute ferric chloride solution is added dropwise to the residue as long as the color deepens. If a red color develops, which is soluble in ether, the presence of cyanide is established.

3. *Prussian Blue Test.*—Twenty cubic centimeters of the distillate are made strongly alkaline with sodium hydroxide solution and concentrated to about 2 c.c. volume. Two drops of 5 per cent ferrous sulfate and 1 drop of 10 per cent ferric chloride are added, and the mixture is slightly warmed. Concentrated hydrochloric acid is then added drop by drop (avoid excess), until the brown precipitate (iron hydroxides) just dissolves. If cyanide is present, a deep blue color or precipitate of Prussian blue remains, which is insoluble in dilute hydrochloric acid.

8. *Silver Test.*—Thirty cubic centimeters or less of the distillate are placed into a 50 c.c. flask, and acidified with 6N nitric acid. A hanging drop of silver nitrate solution on a glass slide is placed over the mouth of the flask. After thirty minutes, the slide is carefully removed, 1 drop of dilute nitric acid is added to the drop on the slide, and a cover slip placed thereon. The material under the cover slip is now slightly heated by applying a very small flame across the underside of the glass slide. On cooling, long slender crystals of silver cyanide are visible under the microscope. Silver chloride crystallizes as octahedra, and can, therefore, easily be differentiated from silver cyanide.

**SULFANILAMIDE, Leucocyte Response to, Bigler, J. A., Clifton, W. M., and Werner, M.** J. A. M. A. **110:** 343, 1938.

Sulfanilamide seemed to cause a depression of the white blood cells, even to a point at which a leucopenia developed.

Agranulocytosis or granulopenia did not occur with this depression or leucopenia.

The action of sulfanilamide seems to be independent of the leucocytes in that it does not produce an increase in the total leucocytes or in the proportion of the polymorphonuclear cells.

Cyanosis occurred in seven cases, but spectroscopic examination of blood specimens in these cases did not reveal bands of sulfhemoglobin or methemoglobin.

Sulfanilamide is a very effective drug in beta-hemolytic streptococcus infections.

Frequent blood cell determinations should accompany sulfanilamide therapy.

**SYPHILIS, Serologic Evidence of, in Malarial Patients, Hazen, H. H., Senear, F. E., Parran, T., Sanford, A. H., Simpson, W. M., and Vonderlehr, R. A.** Arch. Dermat. & Syph. **37:** 431, 1938.

It seems safe to assume that malaria, like leprosy, can be the cause of positive serologic reactions to tests for syphilis. It is probable that more than one patient has been placed under treatment for syphilis when malaria was the cause of his positive serologic reaction. It seems odd that positive reactions are due to diseases caused by such biologically different organisms as an acid-fast bacillus, a spirochete, and a plasmodium. It may be necessary to investigate anew the causation of certain of these diseases, notably that of leprosy.

**CHOLESTEROL Content of the Blood in Heart Disease, Poindexter, C. A., and Bruger, M.** Arch. Int. Med. **61:** 714, 1938.

There is a marked difference between the cholesterol content of the plasma of patients with rheumatic heart disease and that of patients with arteriosclerotic or hypertensive heart disease. Patients with rheumatic heart disease frequently demonstrate hypocholesteremia, although for all the patients as a group, the results lack statistical significance when compared with the cholesterol content of the blood of normal subjects. In contrast is the hypercholesteremia often observed for patients with arteriosclerotic heart disease or hypertensive heart disease manifesting some evidence of arteriosclerosis; for these two groups, however, the increase in the plasma cholesterol value is of sufficient magnitude to be statistically significant. For the most part, there is little or no difference between the ratio of ester to free cholesterol in the three types of heart disease studied.

**SYPHILIS, Diagnosis of, in New-Born Infants, Christie, A. U.** Am. J. Dis. Child. **55:** 979, 1938.

A group of 14 newborn infants of treated syphilitic mothers were studied by a quantitative Wassermann technique. Evidence has been presented to show that in 11 cases positive Wassermann reactions of the blood in the first few months of life were due solely to passive transfer of complement fixing substances from mothers to infants through the placenta. Faber and Black's work has been verified, and additional evidence has been accumulated in support of Fildes' law. In three additional cases studied by this method it was possible to show that infected infants produce their own antibodies and to determine the relation between serologic, clinical, and roentgen response to the syphilitic infection in newborn infants.

This work suggests important reasons for using the quantitative rather than the qualitative or routine Wassermann technique. A decrease in titer of circulating reagin shows that complement fixing antibodies which have passed through the placenta are disappearing from the blood of the infant, so that treatment can be safely withheld. An

increase in titer of circulating reagin means that the infant is manufacturing this substance, which is evidence of the infant's infection. It is apparent that such a technique gives a method of study whereby it is possible to make an early diagnosis and to correlate the clinical as well as the roentgen findings with the serologic response to syphilitic infection.

Fildes made the initial observation that the presence of complement fixing antibodies in the cord blood of the infant is diagnostic not of syphilis in the infant but rather of syphilis in the mother (Fildes' law).

Cruickshank, in 1924, reported 44 infants whose blood had given a strongly positive complement fixation at birth, but which subsequently became negative, in periods of from three weeks to twenty months after birth. He concluded from these findings that the Wassermann reaction of the blood of the newborn infant is of little value in proving the presence of congenital syphilis.

Cooke, in 1922, studied the transmission from mother to child of complement fixing antibodies in tuberculosis; Cooke and Jeans commented in the same year on the similarity which existed in the case of syphilis, as evidenced by infants whose blood was Wassermann positive at birth only to become negative and remain so without treatment of any type.

Lemez, in 1929, confirmed this transitory positivity of the Wassermann reaction and pointed out that should the reaction become negative in the first weeks of life and remain so, the child should be considered syphilis free so long as he remains healthy and his Wassermann reaction remains negative.

Dunham, in 1932, pointed out that this fading out of complement fixing bodies can be measured quantitatively. Roby and Lembcke, in the following year, confirmed this work, stating that with a decreasing Wassermann reaction treatment should be withheld.

**ANAPHYLAXIS, Gangrene Following Administration of Horse Serum, Kohn, J. L., McCabe, E. J., and Brem, J.** Am. J. Dis. Child. 55: 1018, 1938.

A case is presented in which a patient sensitive to horse serum acquired extensive gangrene after repeated injections of horse serum at short intervals at the same site. This reaction may occur, however, when successive injections are made at different sites.

The extensive gangrene, the authors believe, occurs only in the presence of an acute bacterial infection, usually septic sore throat, scarlet fever, or diphtheria.

For the interpretation of this severe reaction in sensitized persons, a factor in addition to the usual antibody-antigen reaction (Arthus's phenomenon) is suggested. The role of the Shwartzman phenomenon in such a reaction is discussed. An important factor of this phenomenon is the presence of a circulating toxin in the patient ill with a concomitant bacterial infection. Thus the combined interaction of fixed horse serum antigen and its antibodies and bacterial toxin at the site of injection may be the cause of extensive hemorrhage and subsequent gangrene.

This intensive reaction, although rare, should remind the physician to be cautious in the administration of repeated injections of horse serum, especially if the patient is sensitive to horse serum.

**VITAMIN C in the Blood, Spinal Fluid and Urine, Wortis, H., Liebmann, J., and Wortis, E.** J.A.M.A. 110: 1896, 1938.

The vitamin C content of the blood, of the spinal fluid, and of the five-hour urinary excretion after a 1 gm. intravenous test dose of cevitamic acid was determined for 133 patients.

1. A blood content of vitamin C above 0.7 mg. per 100 c.c. (by the method of Farmer and Abt) is almost invariably associated with a normal spinal fluid content and a normal urinary excretion test for vitamin C. A blood content of vitamin C below 0.4 mg. per 100 c.c. is almost invariably associated with a subnormal spinal fluid content and a

subnormal urinary excretion test. In these ranges the blood is an adequate and accurate index of the state of vitamin C nutrition.

2. In the intermediate subnormal range for blood (from 0.4 to 0.69 mg. per 100 c.c.) all available tests should be used, including clinical evaluation of the patient.

3. Scurvy is a clinical entity, and its diagnosis cannot be made by vitamin C determinations alone.

**SULFANILAMIDE After Transurethral Prostatectomy, Gaudin, H. J., Zide, H. A., and Thompson, G. J.** J.A.M.A. **110:** 1887, 1938.

A study of the efficacy of sulfanilamide as a urinary antiseptic in the postoperative management of 100 cases of transurethral resection has been made.

A control series of a similar number of cases was studied concurrently.

The authors' experience has not demonstrated a sound basis for administration of sulfanilamide in routine postoperative management of these cases. In fact, the unfavorable reactions from it may hinder convalescence.

It is their opinion that the results of its administration in other types of surgical cases, wherein the drug is expected to act as a urinary antiseptic, will be similar to theirs. Its routine use, therefore, seems unwarranted.

**PREGNANCY, Observations on the Etiology of the Toxemias of, Strauss, M. B.** Am. J. M. Sc. **195:** 723, 1938.

Whether there is manifest edema or not, water retention is nevertheless present in greater or less degree in a large proportion of women during the last trimester of pregnancy. It may amount to as much as 10 per cent of body weight.

This water retention is primarily conditioned by the level of the plasma proteins. The amount of water retained is in inverse proportion to the osmotic pressure of the plasma proteins.

Unless the plasma proteins are below a certain level, a regime consisting of 1,500 c.c. of skimmed milk daily, and hence extremely low in sodium but relatively high in calcium and potassium, will result in the elimination of retained water.

**SEDIMENTATION, Blood, Influence of Anemia on, Cutler, J. W., Park, F. R., and Herr, B. S.** Am. J. M. Sc. **195:** 734, 1938.

The present study is a critical appraisal of the scientific basis for the theory that anemia plays an important role in the sedimentation of erythrocytes. Consequently, the justification for current efforts to correct for anemia is considered. The study leads to the following conclusions:

Anemia has little to do with the phenomenon of blood sedimentation. Rapid settling is the result of the red cells forming large aggregates or rouleaux. If no aggregation takes place, sedimentation is slow no matter how marked the anemia.

The ability of the red cells to form aggregates is a function of the plasma and is specific for that plasma. The specificity is little influenced by the size, shape, or number of cells in suspension.

Once aggregates form, they settle at a given rate of speed, more or less in accordance with Stokes' law of hydrodynamics. The greater the mass of the aggregate, the more rapidly does settling take place and vice versa.

The current practice of letting a single determination at the end of an arbitrary time interval, such as one hour, express blood sedimentation fails to study critically the sedimentation phenomenon and leads to erroneous conclusions.

When multiple readings are employed, the nature of the graph isolates the anemia factor (packing phase) from blood sedimentation (aggregation and sedimentation phases). The shape of the curve portrays disease, the index reflects anemia as well.

One cannot correct for anemia and still get consistent sedimentation findings. The very removal of plasma in an endeavor to correct for anemia interferes with the mechanism of rouleaux formation and sedimentation becomes slow in proportion to the amount of plasma removed.

When considerable plasma is removed, the laboratory may report normal sedimentation findings which do not check at all with the clinical state of the patient, a phenomenon referred to in the literature as "overcorrection."

**GLUCOSE TOLERANCE, Manipulation of, by Diet, McCullogh, E. P., and Johnston, C. R. K.** Am. J. M. Sc. **195**: 773, 1938.

The glucose tolerance test has been used to demonstrate the change in tolerance which occurs following the use of various diets.

Two groups of cases are presented: one, a group of 7 individuals with chronic hypoglycemia; and the other, a group of 5 patients classed as mild diabetes mellitus.

In the cases of chronic hypoglycemia, there was a consistent decrease in tolerance following the use of low carbohydrate, high fat diets. Subsequently, in two of these cases increased tolerance followed the use of high carbohydrate, low fat diets.

In the cases of mild diabetes mellitus the tests showed an increased tolerance following the use of high carbohydrate, low fat diets. In two cases, the original diminished tolerance shown in our data followed the use of restricted carbohydrate intake for several years. In one of these, the tolerance gained following the use of a high carbohydrate intake was largely lost one month after the institution of a low carbohydrate diet of equal caloric value.

Since tolerance of glucose is altered so definitely by various diets, a knowledge of dietary habits is necessary for proper clinical evaluation of the glucose tolerance test.

**BLOOD PROTEINS in Delirium Tremens, Thomas, J. M., Semrad, E. V., and Schwab, R. M.** Am. J. M. Sc. **195**: 820, 1938.

The total serum protein, serum albumin, and serum globulin of 24 patients with delirium tremens were determined on admission to the hospital and after the acute delirious phase of the psychosis had subsided.

The serum albumin of 18 cases was below 4 gm. per cent per 100 c.c. of blood on admission. On discharge the serum albumin of 22 cases was below 4 gm. per cent per 100 c.c. of blood, the albumin value of 12 of these 22 cases being lower than it was on admission.

There was no significant alteration in the serum globulin values either on admission or at the time of discharge.

**TISSUE: Early Epidermal Neoplasia, Sutton, R. L., Jr.** Arch. Dermat. & Syph. **37**: 738, 1938.

Cancer is a cellular disease. It begins probably in one cell, the progeny of which form a colony of cells, which is the cancer. A solitary cell may possess all the attributes of cancerousness within itself. Histologic evidence shows that carcinoma of the skin in its earliest stages appears to have originated in one cell.

Neoplastic lesions of the epidermis may be classified in accordance with a slow or swift growth of the altered cells and their strong coherence, weak coherence, or lack of coherence.

Type I (a).—Strong coherence and slow rate of growth produce senile keratosis, hornlike and verrucous lesions or leucoplakia on mucous membranes, wherein the neoplastic cells closely resemble normal ones and slowly replace them by forming a multilayered disk, the expansion of which results in warping and buckling and produces a warty structure.

Type I (b).—Strong coherence and rapid rate of growth produce carcinoma with a comparatively slight tendency to metastasize and in which the epithelium of "pearls" is multilayered.

Type II (a).—Weak coherence and slow rate of growth produce senile keratoses that scale off readily or erythroplakia on mucous membranes and result in intraepidermal and subepidermal neoplasia by the spreading of sheets of cells that undergrow the normal ones and substitute themselves for them.

Type II (b).—Weak coherence and fast rate of growth produce rapidly progressive senile keratoses which become carcinomas of intermediate malignancy and in which the epithelial periphery of pearls tends to be of few layers.

Type III (a).—No coherence and slow rate of growth produce intraepithelial neoplasms of Bowen's and Paget's types and senile keratoses, wherein cells permeate the normal layers and insinuate themselves singly or in small groups among and between normal cells of skin or mucous membrane.

Type III (b).—No coherence and rapid rate of growth produce rapidly expanding intraepidermal clumps of neoplastic cells, which soon invade the dermis in isolated units, forming carcinomas which metastasize readily.

All neoplasms of the skin in which the cells resemble those of the Malpighian layer in undergoing keratinization form a single set, the clinical subdivisions of which are determined by the degree of coherence and the rate of growth.

This theory does away completely with the concept of "precancerosis"; there is no such thing. Small lesions are neoplastic or not neoplastic; if neoplastic they may grow slowly and be benign or grow fast and be malignant. They should all be destroyed.

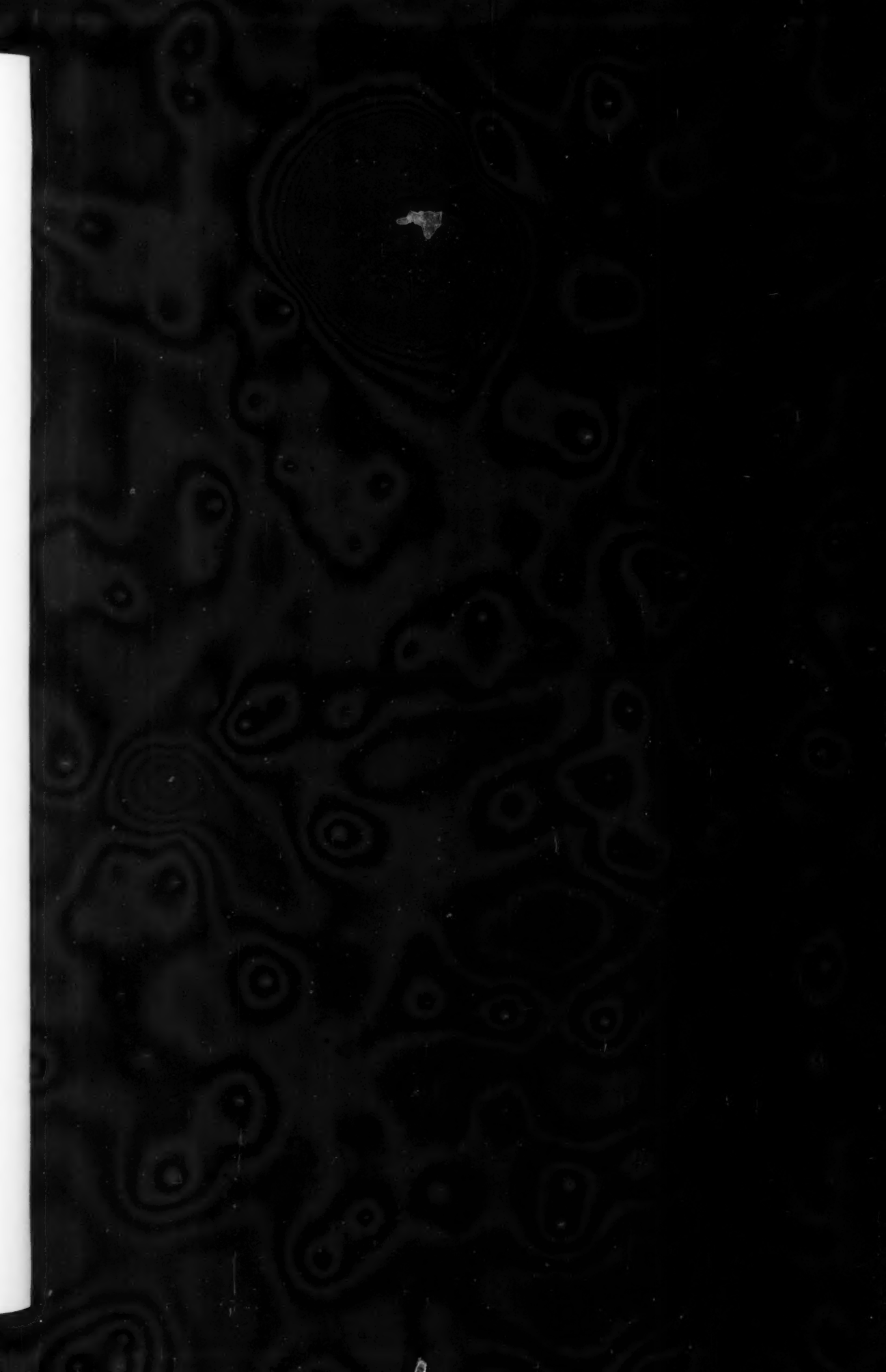
The cells of the keratinizing series of cutaneous tumors have a rate of proliferation exceeding normal and differ from normal in appearance, behavior, and, probably, genetic constitution. The normal rate of cellular reproduction in the human epidermis approximates one mitosis per day for each basal cell, a total of some 60,000,000,000 mitoses daily. Mutation of one of these cells may initiate and explain the progress and properties of epidermal neoplasia.

The theory of the origin of cancer in somatic mutation is outlined and elaborated. This theory postulates that genetic alteration of a somatic cell may result in the production of a colony which is cancer. The new cells differ from normal ones mainly in rate of proliferation and capacity for organization. Neoplasms transferable by filtrable agents are not satisfactorily explained by the theory.

The author's observations show how epidermal carcinoma begins from few cells (probably only one cell) and progresses with no fundamental change excepting continued proliferation. His observations are consonant with the theory of mutation.

**WATER ANALYSIS: Revised Fuchsin Lactose Broth for the Confirmatory Test, Ritter, C.** Am. J. Pub. Health **28**: 616, 1938.

The basic fuchsin content of fuchsin lactose broth should be increased from 0.015 per cent to 0.025 per cent when the broth is to be used as a confirmatory medium in water analysis. Evidence is presented which would indicate that fuchsin lactose broth containing 1 per cent bile and 0.1 per cent dye is also a satisfactory confirmatory medium.



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